Přírodovědecká fakulta

Katedra buněčné biologie

Adaptorové domény signálních proteinů: analýza fosforylačních míst a role v mechanorecepci

Adaptor domains in signalling proteins: phosphorylation analysis and a role in mechanosensing

Diplomová práce

Zuzana Tatárová

Školitel: Mgr. Marian Novotný, Ph.D.

Praha, 2012

Prohlašuji, že jsem diplomovou práci s názvem Adaptorové domény signálních proteinů: analýza fosforylačních míst a role v mechanorecepci zpracovala samostatně, a že jsem uvedla všechny použité zdroje.

I assure that I wrote this diploma thesis called Adaptor domains in signalling proteins: phosphorylation analysis and a role in mechanosensing, myself and that I used only the stated sources and tools.

May, 2012 Zuzana Tatárová

Acknowledgments

First of all, I would like to thank Mgr. Jan Brábek, Ph.D. for letting me be part of the Cancer Cell Invasion Group for the past few years. I would like to especially thank my supervisor Mgr. Marian Novotný, Ph.D. for all his support and constructive comments which greatly improved this work. I am also grateful to all current and past lab members for their help and friendship. My biggest thank goes to my mother, father and two sisters. I am glad to have such a family that gave me valuable advice and support when I needed it the most.

Abstrakt a klíčová slova

P130Cas (Crk-associated substrate, CAS) je důležitým adaptorovým proteinem v integrínové signalizaci, který pozitivně ovlivňuje motilitu, invazivitu, proliferaci a přežívání buněk. CAS postrádá enzymatickou funkci, ale po vazbě jiných signálních proteinu může dojít ke změně fosforylace substrátové domény CAS, což je hlavní způsob, jakým se CAS účastní regulace signálních mechanismů v buňce. Ve fokálních adhezích dochází k lokálnímu pnutí, což vede k natažení substrátové domény, zpřístupnění fosforylačních míst pro kinázy a následně ke zvýšené fosforylaci substrátové domény. Na N-konci je CAS ve fokálních adhezích ukotven pravděpodobně interakcí SH3 domény CAS s kinázou FAK, avšak o ukotvení C-koncové části proteinu CAS není zatím nic známo. Cílem mého projektu je zjistit, jaké proteiny se podílejí na ukotvení proteinu CAS do fokálních adhezí. Objasnění způsobu ukotvení proteinu CAS do fokálních adhezí přispěje k pochopení mechanorecepční funkce proteinu CAS.

Experimentální data poukazují na to, že tyrosinová fosforylace SH3 domény proteinu CAS ovlivňují její vazebné vlastnosti. Druhým cílem mého projektu bylo bioinformaticky analyzovat význam tyrosinové fosforylace SH3 domény a jiných adaptorových domén.

Klíčová slova: p130Cas, adaptorový, CCH doména, SH3 doména, tyrosinová fosforylace

Abstract and Keywords

P130Cas (Crk-associated substrate, CAS) is a multiadaptor protein important in integrin signalling where it positively regulates cell motility, invasion, proliferation and survival. CAS lacks enzymatic activity, but its binding to other signalling proteins could lead to the change of phosphorylation status of its substrate domain, which is the main mode, through which CAS takes part in regulating cell behavior. Local tensions in focal adhesions lead to an extension of CAS substrate domain, leaving phosphorylation sites more accessible for kinases, which subsequently leads to an increased CAS substrate domain phosphorylation. The CAS anchorage in focal adhesions is mediated by its SH3 domain, probably through the interactions with FAK, and also by C-terminal domain, where interaction partners are not known. The aim of my project is to find out, which proteins mediate the CAS anchorage to the focal adhesions. The elucidation of CAS anchorage to focal adhesions will contribute to the understanding of mechanosensory function of CAS.

Experimental data suggest that tyrosine phosphorylation of the CAS SH3 domain plays an important role in the regulation of its binding properties. Another goal of my diploma project was to analyze the significance of tyrosine phosphorylation within SH3 domain and other adaptor domains bioinfomatically.

Key words: p130Cas, adaptor, CCH domain, SH3 domain, tyrosine phosphorylation

List of Shortcuts

aa	amino acids
Ab	antibody
ABC	ammonium bicarbonate
ACN	acetonitrile
app.	approximately
CAS	Crk-associated substrate, p130Cas (protein)
CCH	C-teminal Cas-family homology (domain)
ddH_2O	double-distilled water
ECM	extracellular matrix
FA	focal adhesions
FAK	Focal adhesion kinase
FAT	focal adhesion targeting (domain)
FBS	Fetal bovine serum
GFP	Green fluorescence protein
GST	Gluthation S-transferase
HEF1	Human enhancer of filamentation 1
Hl	Helix long, last 181 amino-acid-region of CAS
Hs	Helix short, last 133 amino-acid-region of CAS
IAA	iodoacetamide
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
MALDI	matrix-assisted laser disorption ionization
MEFs	mouse embryonic fibroblasts

MS	Mass Spectrometry
MSA	Multiple sequence alignment
NSP	Novel SH2 domain-containing protein
O/N	overnight
PBS	Phosphate-buffered saline
PD	Pull Down (assay)
Pfam	Protein Families (database)
pS	phosphorylated serine
PSP	PhosphoSite Plus (database)
pT	phosphorylated threonine
pTyr, pY	phosphorylated tyrosine
SD	substrate domain
SDS-PAGE	sodium-dodecylsulfate polyacryamide (electrophoresis)
SH2	Src-homology 2 (domain)
SH3	Src-homology 3 (domain)
SMART	Simple Modular Architecture Research Tool
RT	room temperature
TBS	Tris-buffered saline
TTBS	Tween tris-buffered saline
wcl	whole cell lysate
WT	wild type
Y12E	phosphomimiking mutation of Y12 of CAS SH3 domain
Y12F	non-phosphorylatable mutation of Y12 of CAS SH3 domain

TABLE OF CONTENT

1 Introduction

Focal adhesions (FA) are cell contacts with extracellular matrix and are important determinants of processes like cell growth (McClay and Ettensohn, 1987), differentiation (Sanes, 1989), development (Ben-Ze'ev et al., 1988), cell adhesion (Evans and Calderwood, 2007), migration and tumorgenesis (Wolf et al., 2003). Bi-directional signalling at these sites are mediated mainly by transmembrane integrin receptor, however, more than 50 proteins have been currently reported to be associated with focal contacts (reviewed in (Zamir and Geiger, 2001)). There is a growing evidence that in cancer cells integrin adaptor proteins are crucial for events resulting in cell transformation and cancer progression (reviewed in (Cabodi et al., 2010)). The molecular mechanism by which these signalling platforms drive the cellular transformation is often not well understood.

Crk-associated substrate (CAS, p130Cas) is one of the adaptor proteins in integrin signalling behaving as a molecular hub for organizing signalling networks in time and space (reviewed in (Defilippi, 2006)). It has been also described as a major mechanosensor in focal adhesions that undergoes phosphorylation in force dependent manner (Sawada et al., 2006). Stretching the adaptor protein p130Cas physically extends its substrate domain making the phosphorylation site more accessible for Src family kinases. This in turn recruits p130Cas partners that promote cell migration by activation small GTPases such as Rap1. Physical extension of p130Cas substrate domain needs anchoring points at two ends of the domain. Early studies showed that focal adhesion targeting domains of p130Cas are Src-homology 3 (SH3) domain at N-terminus (Nakamoto et al., 1997) and Cas-family C-terminal homology (CCH) domain at extreme C-terminus (Harte et al., 2000). Many studies focused on mapping the FA-targeting regions of the protein (Donato, et al., 2010; Harte, et al., 2000; Nakamoto, et al., 1997), however, very little is known about interacting partners of these anchoring domains that would localize p130Cas protein into focal adhesions. The aim of my diploma project was to identify new focal adhesion partners that anchor p130Cas to the cell membrane which will contribute to better understanding of mechanosensory role of CAS. Pull-down (PD) assay and mass spectrometry (MS) analysis was used to determine the physical interaction.

Novel phosphotyrosine sites were identified within SH3 binding surface of CAS and Src. More specifically, Y12 within CAS SH3 domain and homologous Y90 in Src SH3 domain have been reported to be phosphorylated in Src-transformed mouse embryonic fibroblasts (MEFs) (Luo et al., 2008). Our laboratory also contributed to this discovery and focus on biological significance of phosphorylation of these two tyrosines. Thus, we decided to perform bioinformatic analysis that describes abundance and variability of SH3 domain tyrosine phosphorylation. Our results further support recent experimental observations that tyrosine phosphorylation within SH3 domains plays a critical role in the regulation of their function.

2 Review of the Literature

Cancer is a deadly disease mainly because of tumor cells ability to migrate from primary tissue, invade through connective tissues and form metastases at distant location (Kopfstein and Christofori, 2006). Although cell invasion is primarily a mechanical process, cancer research focus mainly on gene regulation and signalling leading to an uncontrolled cell growth (Paszek, et al., 2005; Rolli, et al., 2003; Wolf, et al., 2003). Mechanical processes such as cell adhesion, cell movements and motility, changes of cell shape and generation of forces by cells are still poorly understood. For the understanding of a tumor cell behavior it is important to study it in a context of its microenviroment. Cells sense the structural and mechanical properties of extracellular matrix (ECM). Changes in their close surrounding can lead to adaptation so that cells may generate force from inside to compensate the outside mechanical stimulus. In general, this mechanosensing is mediated mainly by integrin-dependent focal adhesions (FA) and downstream mechanosensor proteins (reviewed in (Brábek et al., 2010).

2.1 Focal adhesions

Focal adhesions are larger, oval, structures located on the periphery of adhesive cells (Figure 2.1). They were first identified by electron microscopy as electrodense regions of plasma membrane that make very close contact between the ventral cell surface and the substratum (Abercrombie et al., 1971). At molecular level, bundles of actin filaments are anchored to transmembrane receptors of integrin family through a complex network of protein-protein interactions. More than 50 proteins were reported within cell-matrix adhesions (reviewed in (Zamir and Geiger, 2001)). Some of them participate as structural links between membrane receptors and actin cytoskeleton, while the others are signalling molecules, including kinases, phosphatases, their substrates and adaptor proteins. Recent challenges are to determine how the numerous associated molecules work together and lead to such a variety of different biological responses.

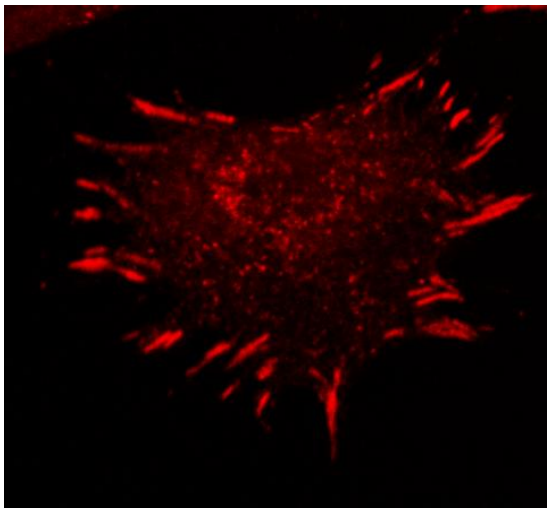


Figure 2.1: Immunofluorescence visualization of focal adhesions in mouse embryonic fibroblasts. The cells were grown on fibronectin and stained with anti-phosphotyrosine to mark FA. Focal adhesions are situated at the cell periphery.

2.1.1 **Focal adhesion components**

Bi-directional signalling at focal sites is mediated by major transmembrane adhesion receptors – integrins. They are heterodimers that are composed of non-covalently associated α and β subunits each of which is a single-pass type I transmembrane protein (reviewed in (Humphries et al., 2006)). Depending on combination of the integrin α and β subunits, 24 distinct integrins receptors can be formed. These distinct receptors can bind ECM proteins such as fibronectin, laminin, collagens and vitronectin with various affinities bind but they can also participate in cell-cell interactions (reviewed in (Hynes, 2002; Luo et al., 2007)). Although cytoplasmic domains of integrins are short several divergent types of proteins were shown to be directly connected to them including chaperone proteins calreticulin and calnexin (Lenter and Vestweber, 1994; Rojiani, et al., 1991), cytoskeletal proteins talin, α-actinin, and filamin (Horwitz, et al., 1986; Otey, et al., 1990; Sharma, et al., 1995) but also regulatory proteins like FAK (focal adhesion kinase), paxilin or integrin linked serine/threonine kinase p59ILK (Hannigan, et al., 1996; Schaller, et al., 1995). Many more proteins are, however, connected to integrins indirectly through interaction with other proteins. The schematic representations of major FA proteins and protein-protein interactions are showed in Figure 2.2. For more detailed review of protein-protein interactions in focal adhesions see Zamir and Geiger, 2001 (Zamir and Geiger, 2001).

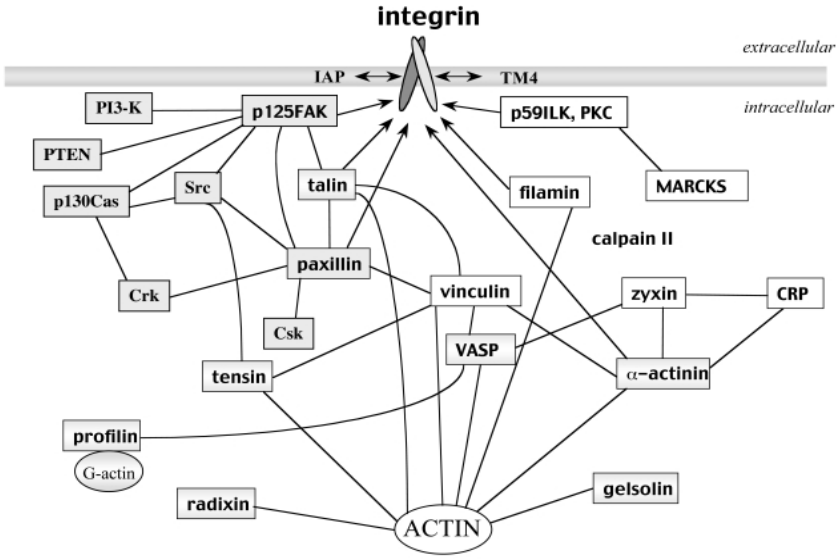


Figure 2.2: Schematic representation of major protein-protein interactions in focal complexes. Lines are presenting direct interactions between the proteins (Petit and Thiery, 2000).

2.1.2 **Focal adhesion signalling**

In addition to their mechanical function in anchorage, integrins transmit signals into the cell (outside-in signalling) providing information about the cell location, microenviroment, state of adhesion and surrounding matrix (rewieved in (Hynes, 2002; Miranti and Brugge, 2002) and By contrast, intracellular signals control the affinity of integrins to ligands and thus regulate the rapid and reversible mechanism of cell adhesion (inside-out signalling) (Calderwood, 2004).

Because integrins do not contain enzymatic or actin binding activities all of the structural and signalling events are presumably mediated by proteins associated with tails of integrins or molecules they trigger. Clustering of integrin receptors, especially β subunit activates non-receptor tyrosine kinase like FAK (Guan and Shalloway, 1992) that leads to increased level of tyrosine phosphorylated proteins at these sites (Bockholt and Burridge, 1993; Burridge et al., 1992; Harte et al., 1996; Nojima et al., 1995; Schlaepfer et al., 1998; Vuori and Ruoslahti, 1995). Tyrosine phosphorylation results in a creation of potential Src-homolgy 2 (SH2) binding sites for other focal adhesion components. This subsequently leads to their recruitment and generation of multiprotein complexes that are important for signalling from focal adhesions (reviewed in (O'Neill et al., 2000). Function of other regulatory proteins such as serine-threonine kinase PKC, lipid kinase PI3K and Receptor-like tyrosine phosphataseα is also controlled by

intergin-mediated adhesion (Podar et al., 2002; Sturge et al., 2002; von Wichert et al., 2003). The integrin-growth factor receptor complexes are established upon integrin clustering and enable crosstalk between the two transmembrane proteins in focal adhesions (Miyamoto et al., 1996; Plopper et al., 1995). Recently adaptor proteins has been highlighted to be important downstream components at this signalling, participating in cellular transformation and cancer cell progression (reviewed in (Cabodi et al., 2010)).

2.1.3 **Focal adhesion assembly**

Focal adhesions are formed of nascent adhesions, so called focal complexes which are dot-like structures of approximately $1\mu m^2$ and are localized within lamelipodium of migrating cells. The mechanism by which the adhesions are nucleated lies in physical clustering of integrin receptors that is followed by incorporation of other components and various signals (Miyamoto et al., 1995). After initiation, focal complexes can break down or evolve into stable focal adhesions depending on forces that are applied to these sites either internally or from outside. Intracellular forces originate from a myosin-II contractile machinery which is mediated by Rho-ROCK signalling (Chrzanowska-Wodnicka and Burridge, 1996). Focal adhesion growth can be induced also by applying external forces which however bypass the requirement for ROCK and myosin-II dependent contractility and activate another target of Rho, mDia, instead (Riveline et al., 2001). The general notion is that contraction puts tension on adhesion molecules and induces changes in their conformation leading to downstream signalling (Sawada et al., 2006). Tension-sensitive molecules contain p130Cas, talin, fibronectin, and integrin (del Rio et al., 2009; Friedland et al., 2009; Sawada et al., 2006; Zhong et al., 1998). Other molecules react to tension by entering or leaving adhesions. Mechanical force mobilize zyxin from adhesions to actin filaments (Yoshigi et al., 2005), and vinculin is recruited to adhesions in response to force (Grashoff et al., 2010).

Hence the key mechanism that induces the formation of focal adhesion assembly is the response to local mechanical forces which underlines the mechanosensory role of focal adhesions and its components.

2.2 P130Cas

p130Cas (Crk-associated substrate, CAS) is a protein of integrin signalling and was first identified as a 130kDa protein that showed significant tyrosine phosphorylation and tight association with v-Crk in cells transformed by onkoproteins v-Src and v-Crk (Sakai et al., 1994). CAS lacks enzymatic activity but having a variety of docking domains it creates a platform for a significant number of interacting partners CAS organizes its interacting partners in time and space which makes this protein one of the most important adaptor proteins in focal adhesions. From N-terminus, it contains a Src-homology 3 (SH3) domain, a large central substrate domain (SD) with multiple potential SH2 binding sites, a serine-rich region, a Src-binding domain (SBD) and a conserved C-terminal Cas-family homology (CCH) domain (Figure 2.3). An unphosphorylated form of CAS localizes in the cytoplasm but upon cell adhesion it move to the cell membrane, becomes highly phosphorylated at central substrate domain which is actually the key signalling event how p130Cas participate in integrin signalling (Abassi et al., 2003; Tachibana et al., 1997).

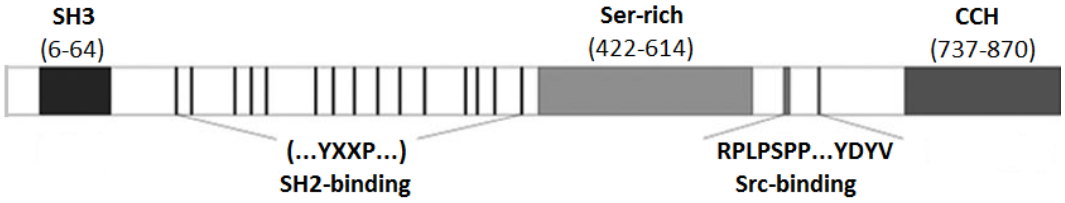


Figure 2.3: Modular structure of p130Cas adaptor protein. Human CAS protein is composed of 870 amino acids and contains several docking domains. These are from N-terminus: Src-homology 3 (SH3) domain, substrate domain with multiple potential SH2 binding sites, serin-rich region, Src-binding domain and a C-terminal Cas-family homology (CCH) domain. Sequence ranges of the domains are written in brackets.

2.2.1 **P130Cas signalling**

CAS substrate domain is composed of 15 YXXP motifs (where X represents any amino acid) that undergo tyrosine phosphorylation mediated mainly by Src or Src family kinases (Klinghoffer et al., 1999; Vuori et al., 1996). In focal adhesions, Src is recruited to CAS either by direct interaction of its SH2 and SH3 domain and CAS Src-binding domain or indirectly through FAK. CAS contains two proline-rich sites (one N-terminal

and one C-terminal) that can potentially bind SH3 domain of Src. Moreover, C-terminal motif includes Y668 (in the motif YDYV) that fits the consensus sequence for high affinity Src SH2 domain. Both pY-668 and C-terminal pro-rich site are known to bind Src (Burnham et al., 1996). FAK binds CAS SH3 domain and when phosphorylated at Y-397 it recruits the SH2 domain of Src (Ruest et al., 2001). In this notion FAK function as scaffold rather than a kinase (Schaller et al., 1999).

CAS becomes tyrosine-phosphorylated in response to many different stimuli: integrin-mediated cell-cell and cell-matrix adhesion (Petruzzelli et al., 1996), activation of B-cell through B-cell receptor engagement (Ingham et al., 1996), various growth factor activation (Ribon and Saltiel, 1996) and stimulation by variety of mitogens (Casamassima and Rozengurt, 1997). Once the CAS is phosphorylated, YXXP becomes docking sites for proteins containing SH2 domains, Crk, Nck and SHIP2 (Klemke et al., 1998; Prasad et al., 2001; Schlaepfer et al., 1997). Thus CAS tyrosine phosphorylation appears to be an integral component in variety of signalling processes.

2.2.1.1 Role of p130Cas in cell migration and adhesion

A FAK-dependent CAS-Crk coupling serves as molecular switch for induction of cell migration (Cary et al., 1998; Honda et al., 1998; Klemke et al., 1998). In the proposed model Crk-Cas complex activates DOCK180 protein, the exchange factor of small Rho GTPase Rac1 (Katoh and Negishi, 2003; Kiyokawa et al., 1998). The active Rac1 stimulate complex Arp2/3 that induces actin polymerization and reorganization (reviewed in (Raftopoulou and Hall, 2004). Rac1 activation further promotes the CAS phosphorylation and CAS-Crk association showing positive feedback loop in an enhancement of membrane protrusion and cell migration (Akakura et al., 2005; Kiyokawa et al., 1998). Bmx/Etk a member of Tec/Btk family of non-receptor kinases has been shown to be coupled to this signalling by interaction with C-terminal region of CAS leading to its increased phosphorylation and Crk complexing. The interaction between CAS SH3 domain and the major protein phosphatase of focal adhesions, PTP-PEST (Garton et al., 1996), is responsible for downregulation and balanced tyrosine phosphorylation of focal componenst and so also for regulation of cell migration (Sastry et al., 2002). By contrast, PTP-1B phosphatase promotes p130Cas-Crk coupling and migration by dephosphorylating Y221 of Crk (Takino et al., 2003).

Another exchange factor, C3G, is targeted to focal adhesions by association with Cas-Crk complex. C3G via proline-rich region interacts with SH3 domain of CAS and Crk and activates Rap1 (Gotoh et al., 1995) and Ras small GTPases, both of which regulate inside-out integrin signalling (Buensuceso and O'Toole, 2000; Zhang et al., 1996). Chat, an NSP family protein can interact with C-terminal part of CAS and increase integrin mediated cell adhesion by activating Rap1 (Sakakibara et al., 2002).

2.2.1.2 Role of p130Cas in cell cycle

It is clear that interaction between CAS and other focal proteins influence cell adhesion and migration. However, several studies suggest that these interactions play role also in cell cycle progression and proliferation. During mitosis when cells are detached from cell substrate, CAS undergoes a dramatic loss of tyrosine phosphorylation but an increase in serine phosphorylation (Yamakita et al., 1999). Furthemore, also FAK-CAS and FAK-Src complexes are also decreased and restored only upon re-entry into G1 phase (reviewed in (Bouton et al., 2001)). A downstream consequence of integrin-mediated cell adhesion is activation of Jun N-terminal kinase (JNK), leading to AP-1 dependent transcription and cell cycle progression. It has been proposed that FAK-Src-Cas-Crk-DOCK-Rac1 pathway is engaged in this process and is regulated by CAS that transmits the signals from ECM and modulates the Src kinase activity (Oktay et al., 1999).

2.2.1.3 Role of p130Cas in cell survival and death

P130Cas is also is known to be a positive modulator of integrin-EGFR crosstalk that leads to cell survival (Moro et al., 2002). It is thought that interaction between CAS SH3 domain and dynamin is responsible for decreased EGFR internalization and so its increased phosphorylation and activation (Kang et al., 2011). There is a direct functional connection between CAS and cell death in generation of dominant negative fragment that actively interfere with survival and cell adhesion signalling. After pro-apoptotic stimulus caspase 9 cleaves p130Cas and generates 31kDa fragment of helix-loop-helix structure that directly heterodimerize with E2A resulting in cell death (Kim et al., 2004; Kook et al., 2000).

2.2.1.4 Role of p130Cas in cell transformation

When considering numerous connections of CAS and the key cellular functions such as adhesion, migration, cell survival and death it is not surprising that this protein is also involved in pathological cell transformation and invasion.

CAS was initially identified as a highly tyrosine phosphorylated protein in c-Src and c-Crk (Sakai et al., 1994). Mutations in v-Src and v-Crk that abrogated interaction with CAS also diminished the transforming ability of these oncoproteins, indicating that CAS plays an important role in process of cell transformation (Kanner et al., 1991). Activated Src mutant expressed in Cas-deficient mouse embryonic fibroblasts was unable to induce anchorage independet growth, full morphogenic transition and full actin filaments assembly into podosomes (Honda et al., 1998). Further study showed that CAS is not a critical determinant of Src-mediated morphologic transformation but its major role is in promoting an invasive phenotype associated with increased podosome formation and activation of matrix metalloproteinase MMP-2 (Brábek et al., 2004). The SH3 domain interactions with DOCK180, CIZ (Cas-interacting zinc-finger protein) and P140Cap were shown to be responsible for regulation of cancer cell behavior (reviewed in (Defilippi et al., 2006)).

CAS also functions in human cancer progression. The human orthologue of CAS (termed BCAR1, breast cancer antigen resistance) was identified in mutagenesis screen as principal gene responsible for resistance to an anti-estrogenic drug, tamoxifen (Brinkman et al., 2000; Dorssers et al., 2004). In human breast cancer cells p130Cas transiently associates with the estrogen receptor in a macromolecular complex together with c-Src. The complex formation is estrogen-dependent and regulates activation of Erk1/2 MAPK and cyclin D1 expression (Cabodi et al., 2004). Further studies revealed a role for adhesion-dependent p130Cas signalling in promoting protein kinase B activation in response to tamoxifen (Soni et al., 2009). So as tyrosine phosphorylation of CAS substrate contribute to invasive behavior of Src-transformed cells (Brábek et al., 2005) it also cause the malignant behavior of mesenchymal like ER-breast cancer cells (Cunningham-Edmondson and Hanks, 2009). The known interacting partners of CAS are schematically presented in Figure 2.4.

Figure 2.4 A domain structure of CAS protein with mapped interacting partners (Tikhmyanova, 2010).

2.2.1.5 P130Cas as mechanosensor in focal adhesions

As it was mentioned at the beginning, CAS plays a central coordinating role in signalling through the tyrosine phosphorylation of its substrate domain. A sequential research on force-mediated signalling revealed that the tyrosine phosphorylation of CAS is implicated also in this important cell process (Sawada et al., 2001; Sawada et al., 2006; Tamada et al., 2004).

Early studies showed that mechanical forces effect intracellular signalling pathways including mitogen activated (MAP) kinases (Sawada et al., 2001). Small GTPases play role in cell adhesion-relevant signal (Gotoh et al., 1995; Sander and Collard, 1999; Tsukamoto et al., 1999) transduction and are also mediators of upstream signalling of MAKP kinase kaskade (Ichijo, 1999). In this context Rap1 small GTPase was shown to be involved in stretch-initiated signal transduction to one of the MAP kinase pathways MKK3/6-p38 (Sawada et al., 2001). To explain the mechanism of the modulation of the small GTPase by stretch Tamada et al. in his study used Triton cytoskeleton which is a remaining cell complex after Triton X-100 extraction composed of few cell membrane and soluble proteins but mainly of cytoskeletal and adhesion proteins. By this approach they showed that mechanical stretch of triton cytoskeleton activated Rap1 and that C3G and Crk bind to triton cytoskeleton in force-dependent manner (Tamada et al., 2004). It was reported before that C3G dependent Rap1-activation is important for adhesion and spreading of mouse embryonic fibroblasts and all three proteins Crk, C3G and Rap1 are part of Src family kinase (SFK) regulated adhesion (Li et al., 2002; Ohba et al., 2001). This led to an assumption that Crk/C3G/Rap1 pathway might be involved in force-induced signalling coming from focal contacts (Tamada et al., 2004). In the same study

the authors showed increased tyrosine phosphorylation of proteins and finally proved that upon cell stretch the main tyrosine phosphorylated protein was CAS. There are four possibilities how cell stretching could increase the tyrosine phosphorylation: 1) Directly by activating kinases, 2) or inactivating phosphatases, 3) mechanically attracting kinase to the substrate or 4) by increased accessibility of the substrate. In 2006, a further study showed that cell stretching enhances SFK-dependent phosphorylation of CAS but without detectable increase in Src kinase activity (Sawada, 2006). As kinase activity did not seem to be a primary mechanism of CAS SD tyrosine phosphorylation the authors prepared bacterially expressed CAS SD protein, CasSD, that was biotinylated on both N-terminal and C-terminal ends and bound to avidin immobilized on latex membrane. By this approach they could mechanically extend the CasSD and prove that increased tyrosine phosphorylation is extension dependent. Using antibody that specifically recognize extended SD of CAS led to an observation that CAS is extended in intact cells in the peripheral regions of spreading cells, where higher traction forces are expected (Geiger and Bershadsky, 2002) and where phosphorylated CAS was detected (Tamada et al., 2004). Thus the authors proposed that p130Cas acts as a primary force sensor, transducing force into mechanical extension of its substrate domain, priming it for increased tyrosine phosphorylation and downstream signalling (Sawada et al., 2006).

2.3 Anchorage mechanism of p130Cas in focal adhesions

For the substrate domain to be extended at focal sites it needs to be anchored at the two ends to the cell membrane. Sawada created a following model: In focal adhesions which are the sites of local tensions p130Cas is anchored to the cell membrane by the SH3 domain at the N-terminus and by a SBD at the C-terminus. Upon cell stretch/adhesion CAS is extended and its substrate domain as well. This makes the tyrosine sites within SD more accessible for Src-family kinases leading to enhanced tyrosine phosphorylation and recruitment of CAS partners that promote cell migration by activating small GTPases such as Rap1 (Figure 2.5).

Later discoveries firmly established that not the SBD but the extreme C-terminal CCH domain is a FA targeting region at the C-terminus of the protein (Donato et al., 2010; Harte et al., 2000).

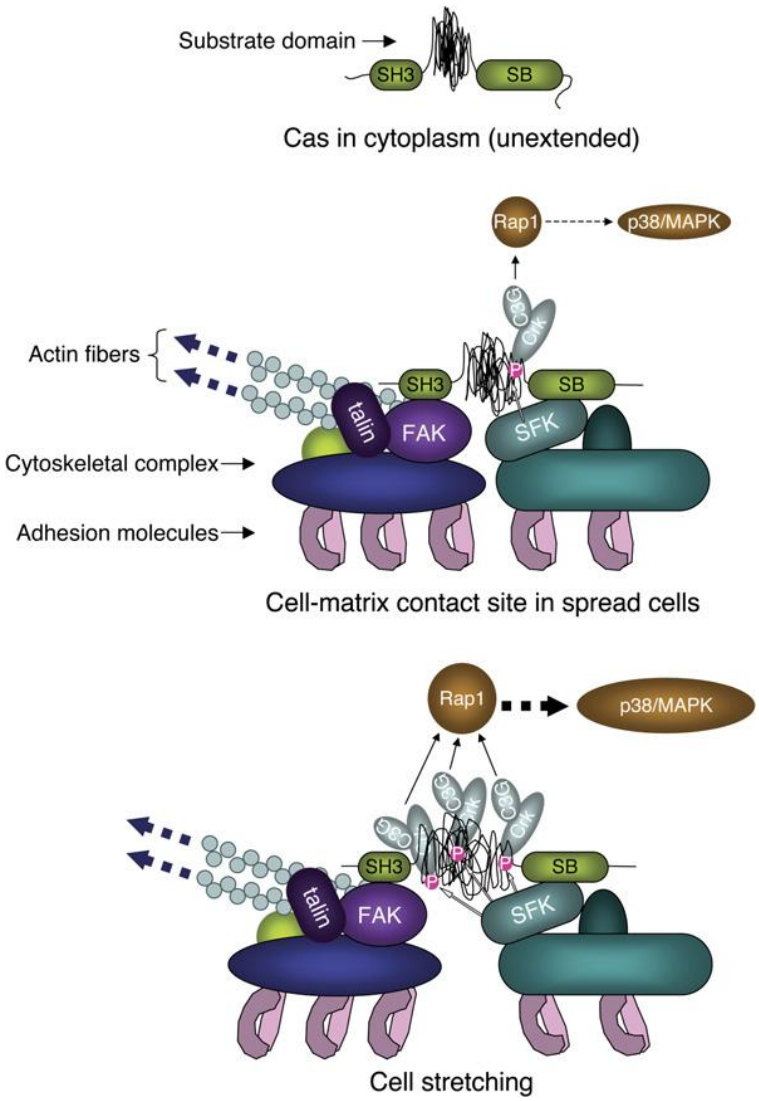


Figure 2.5 Model of mechanosensory role of p130Cas in focal adhesions. CAS mediates its mechanosensory role via tyrosine phosphorylation of its susbtrate domain. The top and the middle panel respresent CAS molecule with CAS substrate domain unextended. The bottom panel shows the force/adhesion-dependent phosphorylation of CAS SD by Src-family kinases that subsequently results in downstream signalling. In the proposed model, the SH3domain at the N-terminus and the Src-binding (SB) domain at the C-terminus are responsible for anchoring CAS into cell membrane (Sawada et al., 2006).

Thus, SH3 domain at N-terminus and CCH domain at C- terminus are the two anchoring points of CAS substrate domain and thus are responsible for mechanosensory role of CAS at cell-matrix contact sites. However, the nature of interacting proteins that target these domains into cell membrane at focal sites is poorly understood (Nakamoto et al., 1997; Polte and Hanks, 1995).

2.3.1 **CCH domain as the C-terminal CAS anchoring domain**

P130Cas along with HEF1 (human enhancer of filamentation) also known as CASL (Crk-associated substrate in lymphocyte) (Law et al., 1996), Efs/Sin (embryonal Fyn substrate/Src interacting (Ishino et al., 1995) and recently described HEPL (HEF1-Efs-p130Cas-like) (Singh et al., 2008) define a family of structurally related proteins. While the CAS family members share structural similarity, they appear to have distinct tissue expression and subcellular localization (reviewed in (O´Neill et al., 2000)). The carboxy-terminus of the CAS family proteins is the most conserved region and early studies predicted this region to be a helix-loop-helix motif (Law et al., 1999). The carboxy-terminal region of CAS is capable of mediating heterodimerization between HEF and CAS *in vitro* (Law et al., 1996), while helix-loop-helix motif is the main determinant of heterodimerization activity (Law et al., 1999). This domain was mapped to be the second focal adhesion targeting region along with SH3 domain ascribing till then unknown function of this domain (Harte et al., 2000; Nakamoto et al., 1997). Consistently with this finding Donato et al., proved the importance of this domain in localizing CAS into focal adhesions, however, the authors emphasize that the CCH domain is unable of this function in the absence of the SH3 domain (Donato et al., 2010).

2.3.1.1 Structure of CAS CCH domain

For long time there was no structural data available on the C-terminal part of the CAS proteins. Recently a new class of adaptor proteins, NSP (Novel SH2 domain-containing protein) was reported to form stable complex with CAS-family members. CAS-binding piece of NSP proteins was proposed to assume a CDC25-like guanine nucleotide exchange factor (GEF) fold and the C-terminal part of CAS proteins a four helix bundle resembling focal adhesion targeting (FAT) domain of FAK. Two recent studies explained the molecular basis of this interaction, one of which resulted in solving its crystal structure (Garron et al., 2009; Mace et al., 2011). Mace et al., used constructs encoding residues 382-703 of NSP and 645-870 of p130Cas. The two proteins formed an extremely tight 1:1 arrangement characterized by extensive binding interface (Figure 2.6A). The CAS C-terminal domain (739-872) was defined by electron density and formed a four-helix bundle characteristic of FAT domain. Extensive hydrophobic inter-

actions in the core of the domain suggest a stable arrangement of these helices (Figure 2.6B). NSP3-CAS interaction at site 1 is accomplished through a binding groove between helices α2 and α3 of the p130Cas FAT domain. This interaction resembles the α2-α3 binding site used by FAK and Pyk2 to bind paxillin (Hoellerer et al., 2003; Lulo et al., 2009). Mutational analysis revealed that L787E, F794R and D797R weakened the *in vitro* association of the p130Cas FAT domain with the NSP3 GEF-like domain, whereas combinations of two mutations virtually abolished the complex formation.

A) B)

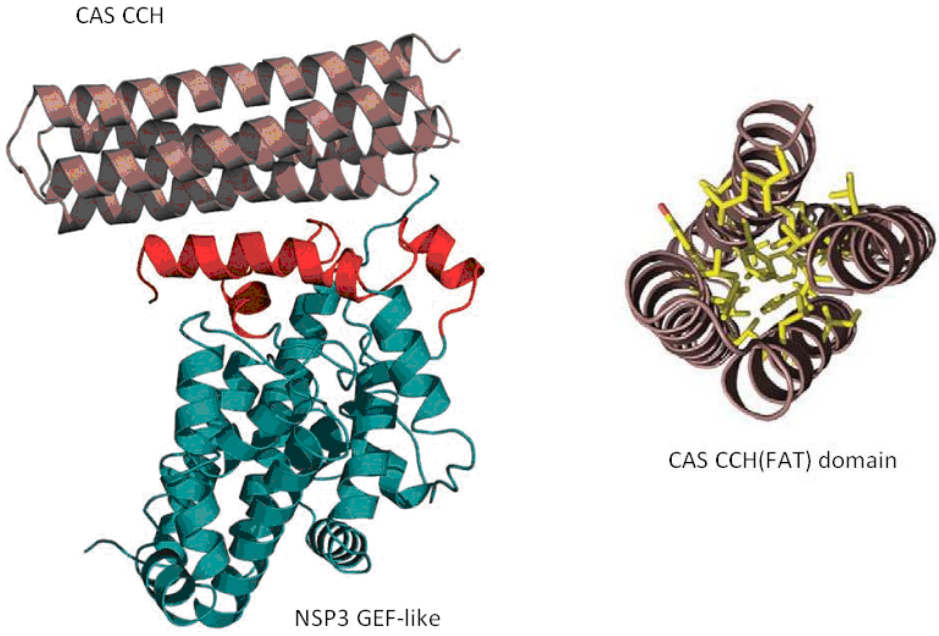


Figure 2.6 Structure of CAS CCH domain. A) Crystal structure of CAS CCH domain (brown) in association with a GEF-like domain of NSP3 protein (cyan). CAS CCH domain adopts a FAT domain four-helix bundle. B) The four helixes create a stable form because of extensive core hydrophobic interactions (colored yellow) (adopted (Mace et al., 2011)).

2.3.1.2 Interacting partners of CAS CCH domain and their effects

There are three members of the NSP family proteins: NSP1, BCAR3/AND-34 and Chat/SHEP1. The first interaction of one of these proteins with CAS CCH domain was described by Sakakibaras group that proposed Chat-CAS complex to be involved in regulation the JNK signalling pathway and membrane ruffling downstream of tyrosine kinases and MAP kinase (Sakakibara and Hattori, 2000). The Chat-CAS complex is also included in control of cell-adhesion via up-regulation of Cas-Crk-C3G signalling

and activation of small GTPase Rap1 (Sakakibara et al., 2002). *In vivo* analysis also confirmed that Chat/SHEP1 is required for proper CAS-Crk mediated signalling (Roselli et al., 2010).

AND-34 is a murine NSP protein that binds by GEF carboxy-terminal to CCH domain of CAS. Over-expression of both of the proteins, the human homolog of AND-34, BCAR3, or the human homolog of p130Cas, BCAR1, induces resistance to growth inhibitory effects of anti-estrogens such as tamoxifen in estradiol-dependent human breast cancer cell lines (Brinkman et al., 2000; Cai et al., 2003; van Agthoven et al., 1998). BCAR3 was also shown to function synergistically with CAS to enhance Src activation and cell migration by re-localizing CAS from focal adhesions to lamellipodia located at the leading edge of migrating cells (Riggins et al., 2003). By contrast CAS functions as a negative regulator in AND-34 guanine nucleotide exchange activity towards Ral small GTPase (Gotoh et al., 2000). Recently BCAR3 was demonstrated to regulate p130Cas serine phosphorylation that is adhesion-dependent which is very distinct from well-characterized rapid FAK and Src kinase-mediated p130Cas tyrosine phosphorylation (Makkinje et al., 2009). BCAR3 further regulates the interaction between CAS and c-Src, both qualitatively as well as quantitatively. The coordinated activity of these proteins contributes to breast cancer cell adhesion signalling and spreading proposing that the c-Src/CAS/BCAR3 signalling axis is a prominent regulator of c-Src activity (Schuh et al., 2010).

The Ajuba/Zyxin family of LIM proteins is a component of focal adhesions and was shown to be involved in regulation if cell migration but not cell adhesion, spreading and integrin activation (Pratt et al., 2005). In the same study the authors ascribed that Ajuba binds C-terminal region of CAS and is upstream of CAS suggesting that it regulates cell motility by activating Rac through recruitment of p130Cas to nascent adhesion sites (Pratt et al., 2005).

In 2004, a novel CAS-interacting protein P140Cap (CAS-associated protein) was described as a new tyrosine phosphorylated molecule involved in integrin- and epidermal growth factor (EGF)-dependent signalling. The protein negatively regulates Src-dependent migration and invasion and impaires integrin-dependent p130Cas phosphorylation, leading to inhibition of Rac1 (Di Stefano et al., 2007). The interaction has been mapped to C-terminal part of CAS but also including consensus sequences for SH3 and SH2 domains (Di Stefano et al., 2004).

Thus several studies described CCH domain in association with another protein. Neither of them, however, is a proper candidate for targeting CAS to FA through the CCH domain.

2.3.2 **SH3 domain as the N-terminal CAS anchoring domain**

The SH3 domain became a prototype of a domain family that share a common feature of specific proline-rich motif binding and till now is one of the most well characterizes protein-interaction modules. SH3 domain-mediated signalling is involved in wide variety of biological processes ranging from regulation of enzyme through intermolecular interaction, recruitment interacting partners to membrane cell parts and generally mediating the assembly of large multiprotein complexes. The SH3 domains are composed of approximately 60 amino acids and share a common secondary β-structure connected by loops (reviewed in (Kaneko et al., 2008)).

2.3.2.1 Structure of CAS SH3 domain

In 2005, a high-resolution X-ray structure of the human recombinant CAS SH3 domain was determined (Wisniewska et al., 2005). As a homolog of the corresponding Src SH3 domain it assumes similar structure (Yu et al., 1992). The SH3 domain of CAS is made of five antiparallel β-strands (sequentially β1, β2, β3, β4, β5) to the standard definition of the SH3 domain region with additional C-terminal β6 strand. The strands are arranged as two orthogonal β-sheets packed against each other at approximately right angle. One of them is formed by β1 and β5 and the other by β2, β3 and β4. First two strands, β1 and β2 are linked by RT loop (named by R10, T25 residues find in Src SH3 domain), which forms a hairpin-like structure. The structure of the RT-loop is stabilized by intra-loop hydrogen bonds and loop-protein core hydrogen bonds. The most highly conserved residues are located on the RT loop (Tyr10 and Glu19) and are involved in protein-peptide ligand interactions. The second strand β2 is linked with β3 strand by intervening short n-src-loop and along with RT-loop is situated on the ligand binding face of the domain and is crucial for modulation of protein–ligand interactions. Distal-loop (between β3 and β4) is situated on the opposite site of the domain and its role is unknown (Figure 2.7A). The strands as well as lops which connect them are stabilized

by network of hydrogen bonds (Wisniewska et al., 2005). The hydrophobic peptide ligand binding cleft of CAS SH3 domain is formed by notably conserved residues, namely Lys9, Tyr10, Gln19, Trp41, Pro54.

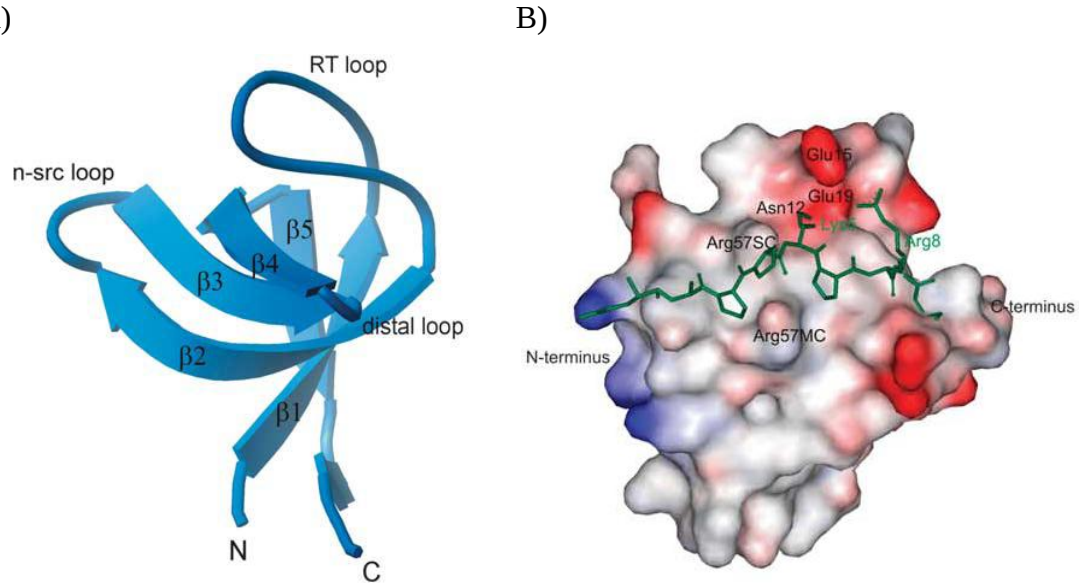


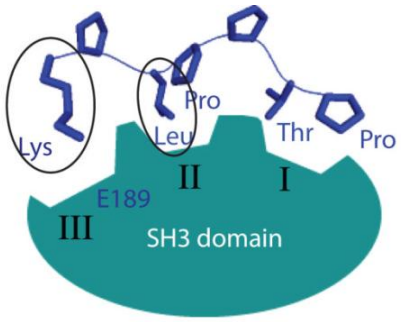
Figure 2.7 Structure of CAS SH3 domain. A) A ribbon representation of CAS SH3 domain. Globular structure is created of two perpendicular antiparallel β-sheets made of three β-strands each (β2, β1, β5 and β2, β3, β4). First two strands, β1 and β2 are linked by RT loop. n-src-loop links the strand β2 with β3 and distal loop is situated on the opposite side of the domain. B) Model of FAK peptide bound to CAS SH3 domain. The SH3 domain interface is colored in red (negatively charged residues), blue (positively charged residues). The FAK peptide is green (adopted (Wisniewska et al., 2005)).

2.3.2.2 SH3 domain ligand-binding surface

Ligand binding surface of SH3 domain is relatively flat and hydrophobic consisting of three shallow pockets (grooves) (Figure 2.8A). Mutational analysis of Src SH3 domain revealed requirement for the conserved amino acid residues in these three pockets. The first pocket is formed in the Src SH3 domain by the side chains of Y92, W118, P133 and Y136, and the second pocket by Y90 and Y136 (numbering for chicken Src). The third pocked is created of Y131 and W118 while the Y131 mutation did not affect the SH3 domain binding properties as much as other mutation of these hydrophobic residues (Erpel et al., 1995). The SH3 domain interacting partners can adopt polyproline type helix conformation, termed polyproline-2 (PPII) helix that is recognized by binding surface of CAS SH3 domain. Generally, peptides that have this conformation are extended and are able to fit the grooves of the SH3 domain ligand binding sites (Yu et

al., 1994; Mayer, 2001). The helix has three residues per turn, in cross-section is roughly triangular and thus the base of this triangle sits on the surface of the domain (Figure 2.8B).

A)



B)

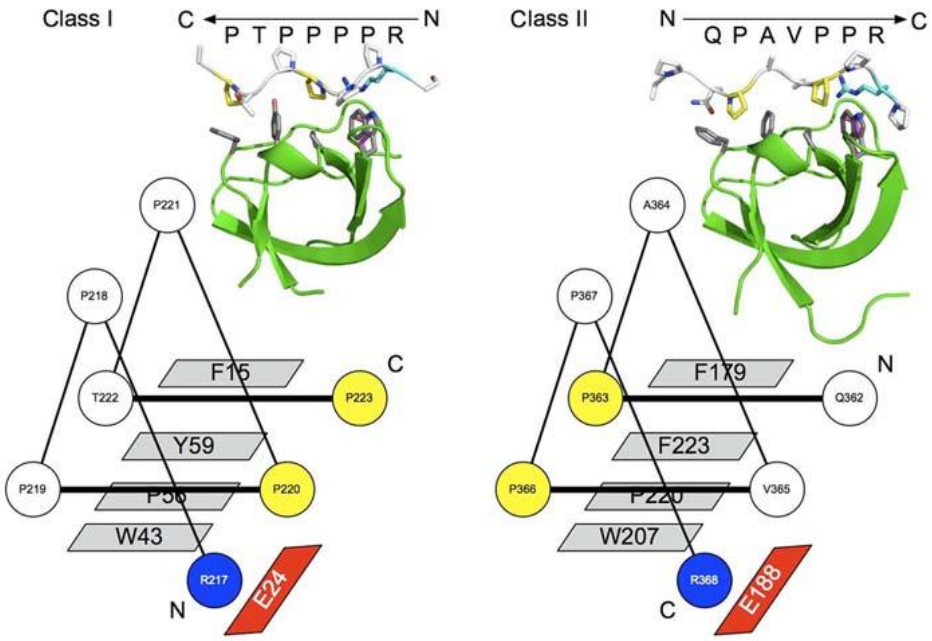


Figure 2.8 Binding surface of CAS SH3 domain. A) Schematic presentation of classical SH3/ligand interaction. A proline rich ligand fits into the relatively flat, hydrophobic SH3 binding surface which is made of three grooves labelled here as I, II and III (adopted (Severin et al., 2009)). B) The proline-rich peptide creates a left-handed helix and in cross-section is roughly triangular. There are two different classes of polyproline-rich ligands. Type I ligand binds to SH3 domain in carboxy to amino (C to N) orientation while the type II has the positively charged amino acid at the C terminus and bind to the groove in N to C orientation (Kaneko et al., 2008).

A ligand can bind to SH3 domain in two orientations depending on position of possitively charged aminoacid (usually arginine) relatively to invariant prolines: Class I ligands with general consensus +xΦPxΦP and class II with ΦPxΦPx+ (reviewed in (Kaneko et al., 2008)). While prolines bind first two pockets of an SH3 domain, the

positively charged arginine interacts with the third pocket and determine the directionality but also the specificity of the interaction (third pocket is also called as specificity pocket.) The ΦP dipeptides are the basic residues responsible for invariantness and give almost the all of the binding energy. Lim and colleagues showed that the requirements for ΦP dipeptides are due to the unique structure of proline as this is the only N-substituated aa. The specificity of the ligand binding may result from the third specificity pocket of the domain (Wu et al., 1995) or ligand residues flanking the core binding motif that are unique to individual SH3 domains and bind to SH3 domain surface but outside ΦP binding groove, such as the highly variable RT and n-Src loop (Feng et al., 1995; Sparks et al., 1996). An example of this notion is in a Src family kinase, Hck, which contain a single residue in the SH3 domain RT-loop with high affinity of binding native Nef but not the isolated pro-rich peptide thus illustrating that PPII distant regions can modulate the ligand binding (Lee et al., 1995).

2.3.2.2.1 Ligand-binding surface of CAS SH3 domain

CAS SH3 domain mediates its interaction with several proteins involved in signalling pathways such as focal adhesion kinase (FAK), tyrosine phosphatases PTP1B and PTP-PEST, guanine nucleotide exchange factor C3G (reviewed in (Defilippi et al., 2006)). Natural ligands of CAS are of the type II, have the positively charged amino acid at the C terminus and bind to the groove in N to C orientation. With the known structure of CAS SH3 domain Wisniewska et al. were able to model the docking interaction of SH3 domain to its natural interactor FAK (Figure 2.7B). The used peptide had consensus EAPPKPSRP. They also showed that conserved Trp at position 41 is critical for ligand binding. Longer ligands were reported to bind to SH3 domain with higher affinity indicating that flanking amino acids are also important for protein-peptide interactions. The architecture of CAS SH3 domain binding surface is the same as for other SH3 domains but its chemical character is not. It is uniquely polar because of exchange of conserved tyrosine (phenylalanine) at position 12 (ALYD**N**) to asparagines and to arginine at position 57. Glu15 seems to compensate the positive charge of N57 and enables the binding of unusual PxKP ligand sequence. In addition to the hydrophobic contacts mediated mainly by prolines, at the N-terminal side the FAK binding peptide, an intermolecular hydrogen bond exists between the side chain of Tyr10 and backbone carbonyl of Glu FP1 of the FAK peptide. Specificity for the binding partner is created

mainly by the third pocked. This is formed of Trp41, negatively charged Glu19, Ser16 while in proposed model Arg8 of FAK peptide makes a salt bridge with conserved Glu19 (Wisniewska et al., 2005). The orienting salt bridge was found to be the key specificity determinant for cellular proteins binding to SH3 domains (Weng et al., 1995). The model of FAK peptide bound to the CAS SH3 domain according to Wisniewska et al. is shown in Figure 2.7B.

2.3.2.3 Interacting partners of CAS SH3 domain and their effect.

When compared to the C-terminal CCH domain, SH3 domain was firmly mapped as a focal targeting domain at the N-terminus throughout all studies (Donato et al., 2010; Harte et al., 2000; Nakamoto et al., 1997). As described in details before, the SH3 and its localization into FA is mostly important for understanding the mechanosensory role of CAS. However, the other, comparably important role of the SH3 domain is related to substrate domain phosphorylation which is a key signalling switch for downstream pathways from CAS. CAS SH3 domain can either bind to protein tyrosine kinases or to tyrosine phosphatases that highlight the additional role of this domain in regulating the phosphorylation status of CAS SD (reviewed in (Defilippi et al., 2006).

The crucial discovery either for CAS targeting into FA or for CAS SD tyrosine phosphorylation was the identification of FAK as an interacting partner of CAS SH3 domain (Polte and Hanks, 1995). Nakamoto et al. used deletion mutants to identify the CAS focal adhesion-targeting domains. They showed that the SH3 domain of CAS in essential for its localization into focal adhesions and postulated that the association with FAK directs CAS into FA. On the other hand, CAS SH3 domain was localized to FA in FAK-negative cells indicating that FAK is not essential for the localization of CAS and some other molecules interact with the SH3 domain and target CAS to FA. It is also possible that other FAK family members are responsible for localization of CAS into FA (Nakamoto et al., 1997).

The proline-rich region of FAK (amino acids 712-718) has been mapped as a binding site for the CAS-SH3 domain (Polte and Hanks, 1995). Mutation of two critical prolines P712 and P715 to alanines resulted in reduced CAS association which correlated with decreased tyrosine phosphorylation of CAS-SD and CAS/FAK promoted cell migration. The *in vitro* kinase assays showed no difference in kinase activity between FAK

P712/715A mutant and FAK wild type proposing that the decreased phosphorylation of CAS-SD was a result of decreased CAS/FAK association (Cary et al., 1998). Although FAK/CAS interaction correlate with CAS tyrosine phosphorylation FAK is not thought to be the main CAS kinase. The most likely model of CAS SD tyrosine phosphorylation is that FAK and Src cooperate to promote CAS SD phosphorylation in a way where CAS bound via its SH3 domain to FAK becomes phosphorylated by Src bound to the FAK pY-397 site. FAK therefore plays a major role as a docking protein that brings Src to its substrate CAS (Ruest et al., 2001).

CAS is highly phosphorylated by Src and plays critical role in v-Src induced transformation (Honda et al., 1998; Reynolds et al., 1989). It acts downstream of FAK to stimulate cell migration (Cary et al., 1998) and survival (Chan et al., 1999). Biochemical analysis revealed that the CAS SH3 domain selectively inhibited v-Src-stimulated activations of AKT and JNK, but not ERK and STAT3. Attenuation of the AKT pathway by the CAS SH3 domain rendered v-Src-transformed cells susceptible to apoptosis. Inhibition of the JNK pathway by the CAS SH3 domain led to suppression of v-Src-stimulated invasion (Cheng et al., 2004).

PTP-PEST tyrosine phosphatase potentially regulates all CAS related signalling events by dephosphorylation of CAS as it displays a highly restricted substrate preference exclusively for tyrosine phosphorylated p130Cas both *in vitro* and *in vivo* (Garton et al., 1996). The SH3 domain was found to bind a single proline-rich sequence in PTP-PEST and this interaction is essential for recognition of tyrosine-phosphorylated CAS by PTP-PEST (Garton et al., 1997). This underlines the role of CAS-SH3 domain in controlling the phosphorylation status of the CAS protein.

PTP1B associates with CAS via pro-rich sequence on PTP1B and SH3 domain on CAS which results in supression of cell transformation (Liu et al., 1998) and changed cell migration (Takino et al., 2003).

CAS SH3 domain is also responsible for an interaction with guanine exchange factor C3G *in vitro* and *in vivo* (Kirsch et al., 1998). The report clearly demonstrated that CAS-SH3 domain binds to N-terminal region of C3G that harbors a proline-rich CAS-binding site distinct from the Crk-binding site. Mutational analysis revealed the critical role of Lys269 (C3G) within the APPKPPL ligand consensus motif thus showing that SH3 domain of CAS selects peptides sharing the consensus XXPP+PPX (where + represent any positively charged residue) (Kirsch et al., 1998).

CMS (CAS ligand with multiple Src-homology 3 domains), a human homolog of mouse CD2-AP, was identified as a direct interacting partner of CAS-SH3 domain. This cytoplasmic protein co-localizes with F-actin and CAS into membrane ruffles at leading edges of cells suggesting a function as a scaffold protein involved in dynamic regulation of actin cytoskeleton (Kirsch et al., 1999; van Duijn et al., 2010).

Another ligand for CAS SH3 domain was identified by far-Western blotting, CIZ (CAS-interacting zinc-finger protein) (Nakamoto et al., 2000). CIZ shuttles between focal adhesions and nucleus and was reported to bind promoters of matrix metalloproteinases (MMPs). Its overexpression upregulates the expression of MMP-1, -3, -7 (Nakamoto et al., 2000) and MMP- 13 (Shah et al., 2004) and Hsp90β and p130Cas were shown to be involved in this process (Fan et al., 2009).

2.4 Adaptor domains and protein tyrosine phosphorylation

A significant part of protein:protein interactions are mediated by families of protein modules that bind relatively short linear peptides. These protein modules (domains) are defined as conserved, functionally independent protein sequences that in signalling cascades act to connect different components into large complexes (Castagnoli et al., 2004; Hofmann, 1999; Kuriyan and Cowburn, 1997). In multidomain proteins these domains tend to fold independetly most probably because their interface is small and loosely packed or unstructured (Han et al., 2007). These features make protein domains easily identifiable from nucleotide or amino acid sequence that is used by many protein family resources such as Superfamily, SMART, Pfam (Gough et al., 2001; Finn et al., 2010; Letunic et al., 2009; Schultz et al., 1998). Pioneering studies on Src kinase in late 1980s and early 1990s showed for the first time relatively small protein recognition domains to bind short linear peptides (Cicchetti et al., 1992; Mayer et al., 1988; Mayer et al., 1991; Ren et al., 1993; Sadowski et al., 1986). Originally the Src-homolgy 2 (SH2) and 3 (SH3) domains were discovered as homologous parts of proteins but right thereafter they were differentiated into two groups: SH2 as a domain family binding proteins phosphorylated in tyrosines (Mayer et al., 1991) and SH3 domains binding proteins rich in proline residues (Ren et al., 1993). Since then modular domains in proteins like protein-tyrosine kinases, phosphatases or adaptors are thought to have a

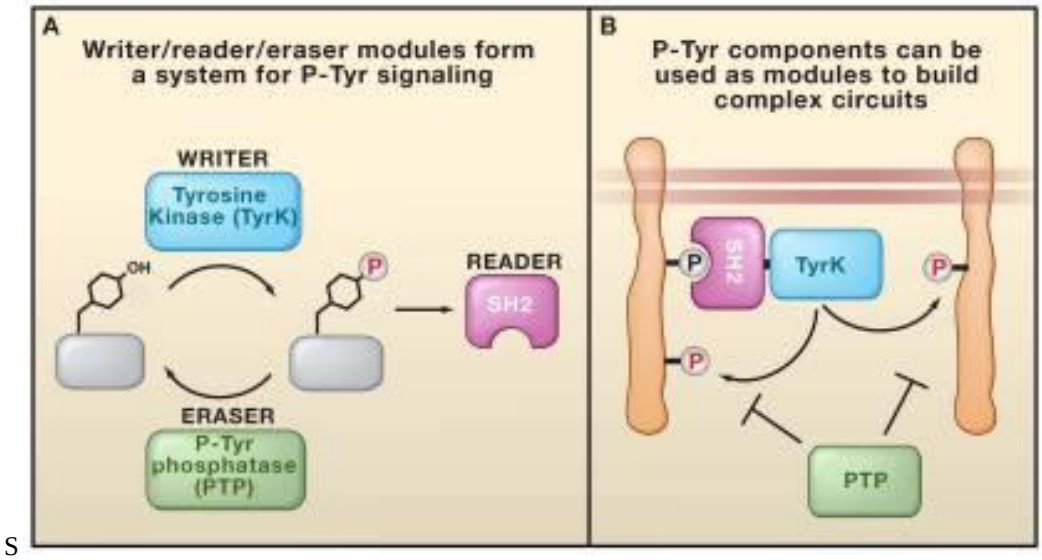
critical role in mediating protein:protein interaction and generation of signals needed for mammalian cell function.

2.4.1 **Protein tyrosine phosphorylation**

Phosphorylation of proteins has been shown to be one of the most central regulatory processes in the cell affecting their intrinsic biological activity, subcellular localization, degradation and docking with other proteins (reviewed in (Cohen, 2000)). Simply defined, phosphorylation is a reversible post-translational protein modification that includes a covalent addition of a phosphate group ($PO4^{2-}$) to an amino acid side chain. The actual impact of $PO4^{2-}$ attachement at the amino acid is three fold: conformational change caused by local/global geometry alteration, change in electrostatic potential and, as a result of both modified protein:target interaction (Alberts et al., 2007). The reaction is energetically unfavorable and thus is catalyzed by enzymes called protein kinases. The protein family of kinases is highly conserved among eukaryotes with three major subfamilies regarding the residue they modify (Ser/Thr, Tyr and dual specificity kinases). Of these three groups protein, tyrosine kinases have not been so far observed in lower eukaryotes (Fungi) and are thought to arose just prior to the evolution of the metazoans (Lim and Pawson, 2010; Ptacek et al., 2005).

Early studies of Rous sarcoma virus transforming protein v-Src and polyoma virus middle T antigen led to the discovery of new type of protein modification, tyrosine phosphorylation (Eckhart et al., 1979; Gilmore et al., 1982; Hunter and Sefton, 1980). In short order, receptor tyrosine kinases (RTK) were shown to be rapidly autophosphorylated on tyrosines upon ligand binding proposing a possible function of tyrosine phosphorylation in mitogene signalling. Phosphorylated tyrosines on activated RTKs are recognizes by phosphotyrosine-binding domain, the SH2 domain. RTKs substrates containing SH2 domain were by this manner recruited to plasma membrane which is essential for downstream signalling (rewieved in (Hunter, 2009; Pawson, 2004)). Till now, this is the best known and thus established model of tyrosine phosphorylation function in cell signalling. On the opposite side to tyrosine kinases there are phosho-tyrosine phosphatases (PTP) that remove the phosphate group from a tyrosine residue and thus together with kinases are responsible for reversibility and dynamics of this process. Together these three modules: tyrosine kinases, “writer”, SH2

domain, “reader” and phoshotyrosine phosphatases, “eraser” create a three part toolkit that is common in distinct cellular platforms and a combination of these three modules can lead to higher-order function (Figure 2.9) (Lim and Pawson, 2010). SH2 domain is not the only domains recognizing pTyr but other domains (PTB, PTP catalytically death domain, C2 domain of PKCα or pyruvate kinase M2) contribute to spreading the complexity mediated by protein-tyrosine phosphorylation (reviewed in (Hunter, 2009)).



S

Figure 2.9 The three part phosphotyrosine signalling toolkit. A three-component system is comprised of tyrosine kinase (writer), tyrosine phosphatase (eraser) and Src homology 2 domains (reader). Components of pTyr are used to build up protein complexes. For example, recruitment of an SH2-PTK to an initiating phosphorylated tyrosine can lead to amplification of tyrosine phosphorylation through a positive feedback loop.

2.4.2 **SH3 domain and tyrosine phosphorylation**

Since Baltimore´s lab described the Abelson SH3 domain recognition of the 3BP1 protein (Ren et al., 1993), extensive pursuit of the varied structures, binding properties, and functions of SH3-containing proteins and the identification of a larger family of domains that recognize proline-rich sequences (i.e., WW, EVH1, GYF domains) have been documented (reviewed in (Zarrinpar et al., 2003)).

An interesting intermolecular interaction has been reported between SH3 and SH2 of Interleukin 2-tyrosine kinase (Itk) protein causing self-association of the kinase. Itk SH3 domain associates with SH2 domain that lacks the proline-rich PXXP sequence defining classical SH3 targets. Neither SH2 domain interaction towards SH3 domain is

phosphotyrosine dependent. Thus, the Itk SH3/SH2 interaction represents an alternative mode of binding for each of these well-studied domains (Brazin et al., 2000; Breheny et al., 2003). On mutational analysis, it was reported that mutation of Y180 of Itk protein to glutamate, used to mimic a phosphorylated tyrosine, leads to a sixfold increase in the affinity of the SH3 domain for Itk (Joseph et al., 2007). Three dimensional structure of Itk SH3/SH2 complex and modeling a phosphate group onto Y180 showed a possible partner residues within SH2 domain of Itk suggesting that intermolecular SH3/SH2 interaction is stabilized up on Itk autophosphorylation within the SH3 (Severin et al., 2009).

By contrast, another member of Tec family protein tyrosine kinases Bruton´s tyrosine kinase (Btk) is autophophorylated at homologous tyrosine site Y223 on the potential ligand binding site (Park et al., 1996). SH3 domain phosphorylation provides a negative charge (and steric exclusion) to this surface that is thought to be severely unfavorable for the interaction with ligands (Hansson et al., 1998). Thus a question is arising: What is the effect of tyrosine phosphorylation within SH3 domain ligand binding surface?

3 Materials

3.1 Bacterial cultivation

Bacterial growth media are prepared of milipore water produced from Watrex unit.

After preparation the media are autoclave-sterilized for 20 min at 121°C.

1× LB (Luria Bertani) medium
10 g/l Universalpepton M66 (MERCK, Germany)
5 g/l Yeast Extract (OXOID, UK)
5 g/l NaCl

2× LB (Super LB)
20 g/l Universalpepton M66 (MERCK, Germany)
10 g/l Yeast Extract (OXOID, UK)
5 g/l NaCl

YTG
5 g/l Yeast extract (OXOID, UK)
8 g/l Tryptone
5 g/l NaCl
1 % Glucose

Bacterial agar
4% živný agar č.2 (Imuna, Šarišske Michalany).

The selection antibiotics are diluted from stock solutions and added to growth media after sterilization when temperature decrease below app. 50°C.
Ampicillin (Biotika, SR) 100 mg/ml (final concentration)
Kanamycin (AppliChem, Germany) 50 mg/ml (final concentration)

3.2 Mammalian tissue culture cultivation

RPMI 1640 (Gibco, Invitrogen) medium is used for cultivation of Hela cell line

DMEM (Dulbecco's modified Eagle's medium) (Gibco, Invitrogen) is used for:
K4 (rat Rous sarcoma RsK4) cells
MEF (mouse embryonic fibroblasts) cells
FAK-/- cells – FAK knockout MEFs
Vcl-/- cells – Vcl knockout MEFs
Cas-/- cells – CAS knockout MEFs
Complete medium contains additional:

0.5% non-essential amino acids (MEM NEAA) (Gibco, Invitrogen)

10% fetal bovine serum (FBS) (Sigma)

0.5% common antibiotics - antimycotics (ATB) (Gibco, Invitrogen)
Contains: 10 mg/ml penicilin
10 mg/ml streptomycin
25 μg/ml amphotericin

Other chemicals used in tissue culture cultivation:

0.25% trypsin-EDTA (Gibco, Invitrogen)
2.5 g/l trypsin
0.38 g/l ethylenediaminetetraacetic acid (EDTA)

Fibronectin (Serva) 1-5 μg/cm^2 working concentration

3.3 DNA manipulation

Ligation of plasmid DNA:
T4 DNA ligase LC (MBI Fermentas, Lithuania), 1 U/μl
10× ligase buffer (MBI Fermentas, Lithuania)

DNA restriction endonucleases:
BamHI (MBI Fermentas)
BglII (MBI Fermentas)
EcoRI (MBI Fermentas)

Kits for DNA purification:
NucleoSpin Extract (Macherey-Nagel, BRD)
NucleoSpin Plasmid (Macherey-Nagel, BRD)

Kit for ligation of PCR products into TOPO vector:
TOPO® TA® cloning Kit (Invitrogen, USA)

3.4 Antibodies

3.4.1 **Primary antibodies**

P130Cas (C-20), sc-860, rabbit polyclonal IgG, (Santa Cruz Biotechnology)

Vinculin (N-19), sc-7649, goat polyclonal IgG, (Santa Cruz Biotechnology)

FAK (C-20), sc-558, rabbit polyclonal IgG, (Santa Cruz Biotechnology)

α Tubulin (B-7), sc-5286, mouse monoclonal IgG, (Santa Cruz Biotechnology)

anti-GFP, A11120, mouse IgG_{2a} monoclonal, (Invitrogen)

GFP (FL) B, sc-8334 B, biotin conjugated , (Santa Cruz Biotechnology)

Anti-Cortactin [pY421], rabbit polyclonal, (Biosource)

Anti-Paxillin610051, mouse MAb (BD Biosciences)

Anti-Phosphotyrosineclone 4G10®, mouse monoclonal IgG_{2b} (MILLIPORE™)

3.4.2 **Secondary antibodies**

Donkey anti-goat IgG-HRP, sc-2033 (Santa Cruz Biotechnology)

Goat anti-rabbit IgG-HRP, sc-2030 (Santa Cruz Biotechnology)

Rabbit Anti-Mouse IgG (H+L) peroxidase-conjugated (ImmunoResearch)

Goat anti-rabbit IgG (H+L), Alexa Fluor® 546 (Invitrogen)

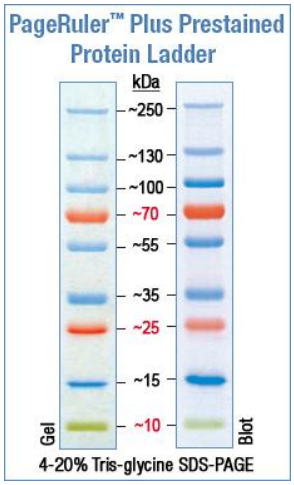
Goat anti-mouse IgG (H+L), Alexa Fluor® 633(Invitrogen)

3.5 General solutions

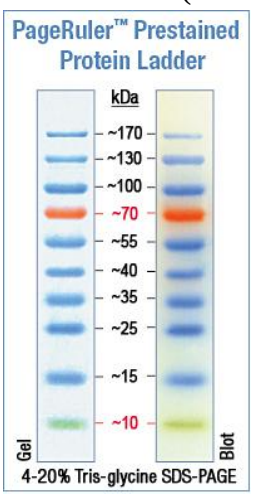
6xLaemmli	10% SDS, 38% glycerol, 62.5mM Tris-HCl (pH 6.8), 0.012% bromphenol blue
10 x PBS	137 mM NaCl, 2.7 mM KCl; 4.3 mM Na_2HPO_4.12 H_2O; 1.4 mM KH_2PO_4; pH 7.3
Ponceau stain	0.04g Ponceau, 2ml EtCOOH (98%) in 40ml of ddH_2O hHwateH222OH2OH

3.6 Protein markers

SM1811



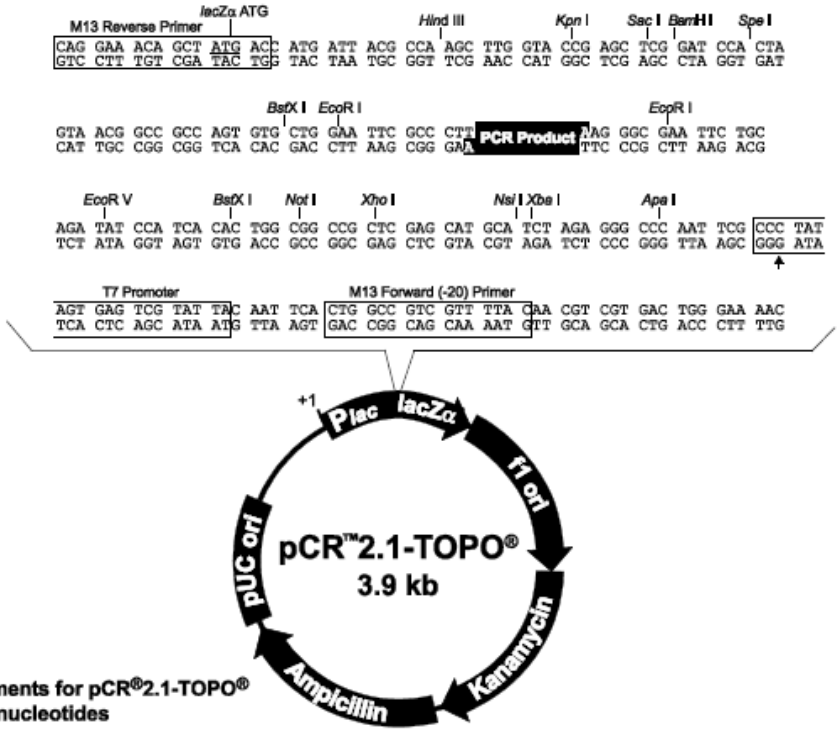
SM0671 (Thermo SCIENTIFIC)



3.7 Vectors

3.7.1 **pCR™2.1-TOPO®**

(used for insertion of PCR products)



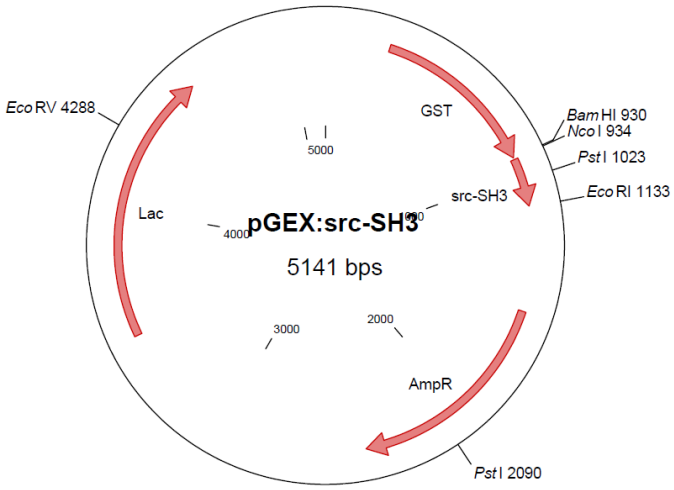
Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

(Invitrogen)

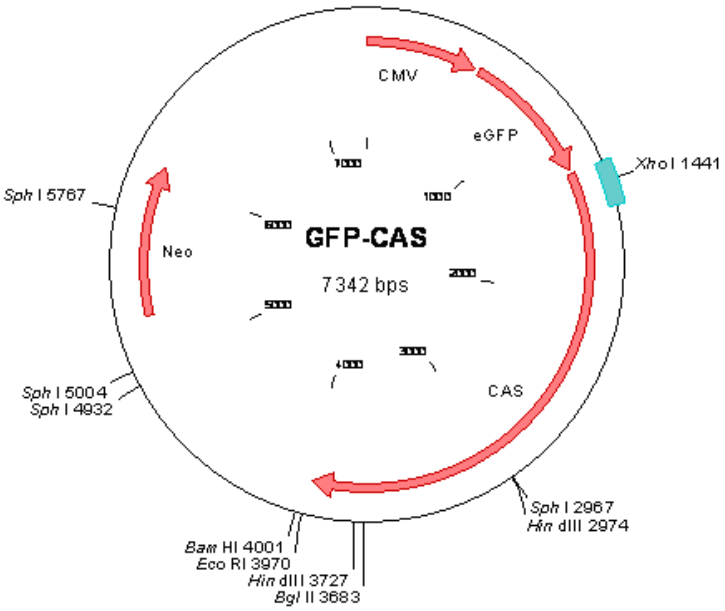
3.7.2 **Bacterial expression plasmid pGEX**

(used for preparation of GST-fused CAS-CCH proteins)



3.7.3 **pEGFP-C1 vector**

(used to for transient transfection of GFP-fused CAS-CCH proteins)



4 Methods

4.1 Standard Methods

4.1.1 **SDS-Polyacryamide (SDS-PAGE) gel electrophoresis**

SDS-PAGE electrophoresis is used to separate proteins according to their electrophoretic mobility. The binding of SDS to polypeptide chain imparts charge according to mass unit thus resulting in fractionation of proteins by approximate size during electrophoresis. Standard SDS-PAGE has a vertical arrangement.

Each piece of equipment used for electrophoresis should be washed and rinsed by distilled water before use. Volumes given are sufficient for small (8cm x 10cm x 0.75 (-1.5) mm) gel format.

- Set up a gel apparatus (see manufacturer's instruction manual) and prepare separating gel monomer (2^{nd} slope in the table):

	10% separating gel	Stacking gel
protogel	1.67 ml	0.325 ml
4xSDS/Tris pH 8,8	1.25 ml	-
4xSDS/Tris pH 6,8	-	0.625 ml
ddH_2O	2.08 ml	1.55 ml
TEMED	13.5 µl	10 µl
APS	66 µl	50 µl

- Immediately pour the separating solution in between the glasses. Before adding the stacking "gel" overlay gently with ddH_2O. Incubate at RT until the gel polymerize.
- Decant the overlay completely and prepare the stacking monomer (3^{rd} slope in table above).
- Immediately insert the comb and allow to polymerize completely before running the gel.
- Load samples that need to be analyzed along with protein marker into the slots.
- Run the gel at constant current for 0.75mm/1mm gel → 10 mA stacking, 20 mA separating per one gel.

- The gel can be used for western blotting or stained with Coomassie blue and used for mass spectrometry analysis.

Protogel	30% akrylamid; 0.8% N,N'-methylenbisakrylamid; filtered over 0.45 µm filter, stored in dark place, 4°C
4x SDS/Tris pH 8.8	1.5 M Tris-HCl (pH 8.8); 0.4% SDS; filtered over 0.45 µm filter, store in dark place, 4°C
4x SDS/Tris pH 6.8	0.5 M Tris-HCl (pH 6.8); 0.4% SDS; filtered over 0.45 µm filter, store in dark, cool place
SDS running buffer	25mM Tris (pH 8.3); 190mM glycine; 0.1% SDS
Coomassie staining	20% methanol; 10% acetic acid; 0.114% Coomassie Brilliant Blue R-250 (Bio-Rad, USA)
Coomassie destaining	5% methanol; 7% acetic acid
APS	25 mg ammonium persulfate in 100ml of ddH_2O

4.1.2 **Western blotting**

Western blotting is used for transferring proteins that were previously separated by SDS-PAGE electrophoresis to a nitrocellulose membrane. Proteins will move to positive charge so be sure to arrange the transfer sandwich so that the proteins move onto the nitrocellulose membrane.

- Prepare the transfer cassette/sandwich by putting:
  - the black piece of transfer cassette
  - one wetted (in transfer buffer) white sponge
  - one wetted Whatman paper sheet (8x10 cm)
  - gel with the separated proteins
  - nitrocellulose membrane (activated by wetting in transfer buffer)
  - one wetted Whatman paper sheet
  - one wetted white sponge
  - roll a 15 ml conical tube on the setup to remove potential bubbles
  - close the cassette
- Place the cassette in a transfer tank (putting black side of sandwich to black side of transfer device).

- Place also ice container and a magnetic stir bar to the transfer apparatus tank to keep the transfer buffer temperature.
- Transfer at 100V for 1.5 hours, with magnetic stirring. Change the ice container for new one in case of need.
- After transfer wash the membrane in TBS.
- The membrane can be stores in TBS at 4°C for up to app. 1 month or can be directly used for immunodetection.

Transfer buffer	25mM Tris; 192mM glycine; 20% methanol; 0.05% SDS
TBS	20 mM Tris-HCl (pH 8.0); 500mM NaCl

4.1.2.1 Chemiluminescent detection of blotted proteins

- Wash the membrane with blotted proteins in TBS for 10 minutes.
- Block the membrane in chosen blocking solution for 60 min. incubate on a rocker (MR-1, Biosan).
- Wash the blocked membrane in large volume of TTBS for 10 minutes.
- Seal the membrane together with primary antibody (Ab) solution and incubate overnight (O/N) at 4°C on the rocker.
- After labeling with the primary Ab, wash the membrane 3 × 10 minutes in large volume of TTBS.
- Seal the membrane with a solution of secondary Ab that is conjugated with horseradish peroxidase. Incubate for 1 hour on the rocker.
- Wash the membrane for 2 × 10 minutes in TTBS and 1 × 10 min in TBS.
- To detect the signal, incubate the membrane for 5 minutes in working solution of SuperSignal West Pico Chemiluminiscent Substrate (Pierce Biotechnology). To prepare the working solution, combine solution of luminol with enhancer and stable peroxide buffer.
- Visualize the signal using a CCD camera (Las 4000, Fujifilm).

TTBS	0.05% Tween 20 (Serva) in TBS
Blocking solution	3% powdered milk (PROMIL) in TBS
Solution for diluting Ab	3% powdered milk in TTBS

4.2 Molecular cloning

4.2.1 **PCR**

The work should be performed carefully with the use of laboratory gloves to avoid the possibility of contamination by external DNA. Also, it is recommended to work on ice to prevent corruption of primers by a premature action of DNA polymerase.

- Prepare 50 µl of PCR mixture as following:

	Volume (µl)
H_2O	33
10X *Taq* Buffer	5
dNTPs (50mM)	0.5
Primer forward (10mM)	5
Primer reverse (10mM)	5
Template DNA (100ng/µl)	1
Tag polymerase (1U/µl)	0.5

- Insert the mixture in a PCR cycler with a preheated lid (Peltier PTC-200, MJ Research, USA) and run the following program:

1. 95°C 2 min
2. 95°C 30 s
3. 56°C 40 s
4. 72°C 1,5 min
5. Go to STEP2, 2x repetitions of steps 2. – 4.
6. 95°C 30 s
7. 63 °C 40 s
8. 72°C 1 min
9. Go to STEP6, 21x repetitions of steps 6. – 8.
10. 72°C 10 min
11. 4°C 5min

10X *Taq* Buffer	750mM Tris-HCl (pH 8.8 at 25°C); 200mM $(NH4)_2SO_4$; 0.1% (v/v) Tween 20; 20mM MgCl2
dNTPs	2.5mM dATP; 2.5mM dGTP; 2.5mM dCTP; 2.5mM dTTP
Taq polymerase	5 U/µl, (Fermentas)

4.2.2 **Agarose gel electrophoresis**

Electrophoresis is performed in horizontal alignment. Distance between electrodes is 14 cm. Length of a gel is 6 cm and thickness app. 5 mm.

- Use fresh 1xTAE buffer to prepare 1 – 2% agarose gel (agarose SeaKem and NuSieve agarose (FMC, USA), 2:1).

Suggested agarose concentrations are:

Size range (bp)	Final agarose (%)
1000 - 23 000	0.6
800 – 10 000	0.8
400 - 8000	1
300 – 7000	1.2
200 – 4000	1.5
100 – 3000	2

- Weight the beaker with solution before heating.
- Heat the powdered agarose in buffer until it melts and dissolves completely.
- Supply distilled water to obtain the initial weight.
- Cool the solution to 50 – 60°C prior to casting.
- Use appropriate comb to prepare slots in the gel and pour the agarose in the resealed mold.
- Load samples containing DNA mixed with 6xloading buffer into the slots. Pipet marker to one of the slots.
- Run the gel by applying voltage 1 – 5 V/cm of the gel. In this case 60 V.
- Transfer the gel into solution containing ethidium bromide and incubate for 10 minutes.
- Wash the gel with distilled water and visualize the DNA with the use of ultraviolet transilluminator.
- The stained gel can be photographed with appropriate digital camera equipped with a red filter in 300 – 310 nm.

1xTAE — 40mM Tris (pH 8.5); 20mM acetic acid; 2mM Na_2EDTA

4.2.3 **DNA fragment isolation with NucleoSpin® Extract**

Use a clean scalpel to cut out the DNA fragment in gel.

- Weigh the gel fragment and for every 100 mg of gel add 200 µl of NT1 solution.
- Dissolve the gel by incubating it at 50°C (5 – 10 min). Vortex every 2 – 3 min.
- Insert a NucleoSpin Extract column into a microfuge tube, load the sample and centrifuge (Hettich MIKRO 20, 8 000 g, 1 min). Discard the flow-through.
- Wash DNA bound to column resin by applying 600 µl of NT3 buffer.
- Elute DNA into a clean microfuge tube with 25 – 50 µl of elution buffer of deionized water. Incubate 1 min at room temperature (RT) and then spin (Hettich MIKRO 20, 11 000 g, 1 min).

4.2.4 **TOPO® TA cloning with TOPO® TA® cloning Kit (Invitrogen USA)**

Do not add 5´phosphatase to primers for PCR. The PCR product synthesized will not ligate to TOPO vector. *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearised vector supplied in the kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

- Set up the TOPO reaction as following:

Reagent	Volume (µl)
PCR product	4
Salt solution	1
TOPO vector	1
Total volume	**6**

- Incubate the reaction for 5 minutes at room temperature
- Transform the TOPO reaction into OneShot® competent cells by adding 2 µl of the reaction.
- Incubate on ice for 5 – 30 minutes.
- Heat – shock the cells for 30 seconds at 42°C, without shaking
- Immediately transfer the cells to ice

- Add 250 µl of Super LB with 1% glucose. Shake the culture at 200 rpm and 37°C for 15 – 60 minutes.
- Spread 20 µl from transformation on a selective plate with ampiciline.
- Incubate in 37°C.
- The next day pick single colony and inoculate 2.5 ml of YTG medium with ampiciline (100mg/ml).
- Grow until culture reaches stationary phase, usually 12 – 16 hours.
- Use cells for TOPO plasmid isolation or store the cells in -80°C (add sterile glycerol to final concentration 10%)

TOPO® vector	by catalog number (Invitrogen)
Strain	by catalog number (Invitrogen)
Salt solution	1.2 M NaCl; 0.06 M $MgCl_2$ (Invitrogen)

4.2.5 **Isolation of plasmid DNA with Nucleospin® Plasmid Kit**

- Pelet (Hettich MIKRO 20, 11 000 x g, 30 s) 2 ml of E. coli strain culture that was grown overnight under ampiciline/kanamycine selection. OD_{600} should be app. 3.
- Discard the supernatant carefully. Resuspend the pelet completely in 250 µl of A1 reagent.
- Add 250 µl of A2 reagent. Mix gently by inverting the tube 6 – 8 times, do not vortex. Incubate for a maximum of 5 min at room temperature.
- Add 300 µl of neutralizing A3 reagent. Mix gently by inverting the tube 6 – 8 times and immediately centrifuge (Hettich MIKRO 20) at 11 000 x g for 5 minutes.
- Place the NucleoSpin Plasmid column in a 2 ml microfuge tube and load on the supernatant.
- Spin the column at 11 000 x g for 1 min to allow DNA binding to silica membrane. Discard flow-through.
- Wash silica membrane with 600 µl of A4 (supplemented with ethanol!) and centrifuge (Hettich MIKRO 20, 11 000 x g, 1 min).
- Discard the flow-through. To dry the column centrifuge for another 2 min.

- Place the NucleoSpin Plasmid column into a new microfuge tube to collect the clear DNA.
- Pipet 30 µl of elution buffer AE onto the silica membrane. Incubate for 1 min at RT. For more efficient elution pre-heat the AE buffer.
- Centrifuge (Hettich MIKRO 20, 11 000 x g, 1 min).
- Transfer collected DNA into new 0.5 microfuge tube and store in -20°C.

4.2.6 **Plasmid DNA restriction with endonucleases**

- Prepare the restriction mixture in 0.5 ml microfuge tube. For 50 µl reaction:

	Volume
DNA sample	(0.1 – 4 ug)
10x restriction buffer	5 µl*
Restriction endonuclease	0.5 or 1 µl*
ddH_2O (deionized water)	Supply to 50 µl

*add restriction endonucleases and restriction buffer as advised at http://www.fermentas.com/en/tools/doubledigest

- Incubate the reaction 3 – 4 hours in 37°C
- Stop the reaction by transfering the mixture into -20°C.
- Examine the amount and quality of cleaved DNA by agarose gel electrophoresis.

4.2.7 **Plasmid DNA ligation**

The molar ratio of vector to insert DNA should be 1:3 (depending on the size of insert when compared to size of vector). Prepare additional ligation mixture without insert DNA (add water instead) as a control of ligation.

- Prepare 12µl of ligation mixture in 0.5 ml microfuge tube:

	Volume
Vector, insert DNA (3:1)	0.1 – 10mM
Ligation buffer	1.2 µl
ddH_2O	Supply to 12µl

T4 DNA ligase	0.2 µl (0, 08U)

- Incubate for 3,5 hours at RT.
- Stop ligation by placing DNA into – 20°C O/N.
- Use 3 µl of the mixture to transform *E. coli* DH5α strain or BL21 strain.

DH5α strain	φ80dlacZΔM15, recA, gyrA96, thi-1, hsdR17 (r_{K}^{-}, m_{K}^{+}), supE44 relA1, deoR, Δ(lacZYA-argF),U,169
BL21	strainF-, ompT, $r_{B}m_{B}$, λDE3 lysogen

4.2.8 **Electroporation of plasmids**

- Thaw electrocompetent cells on ice. Transfer 50 µl of the cell suspension to 0,5ml microfuge tube and mix thoroughly with 3 µl of plasmid DNA.
- Add the mixture to pre-cooled 0.2 cm electroporation cuvette. On a pulse generator - Gene Pulser Apparatus (Bio-Rad, USA) adjust the following settings: capacitance 25 µF, voltage 2.5 kV and resistance 200 Ω.
- Insert the cuvette into the pulser and apply puls 12.5 kV/cm. The resulting time constant should be in the range 4 – 5 miliseconds.
- Immediately add 1xLB medium with 1% glucose to the cell mixture.
- Resuspend the cells and transfer them to a 10 ml glass tube.
- Shake the transformed cell culture at 200rpm and 37°C for at least 45 minutes.
- Spread 20 µl and 200 µl from each transformation on two selective plates with ampicilline/kanamycine.
- Continue as described before 4.2.4

4.3 Cell culturing

Cell cultures are examined daily (microscope Nikon Eclipse TS 2000), observing the morphology, the color of the media, the density of the cells and contaminations in case. Mammalian cells are cultivated in an incubator with 5% CO_2 at 37°C on culture dishes Ø 60 mm (or Ø100 mm respectively). The amount of growth medium is 4 ml for small Ø60 mm dishes or 10 ml for Ø100 mm dishes. The cells are passed every time when

they reach confluence, usually every 3rd or 4th day. Before use pre-heat the growth medium and trypsin for 15 min (preferably not more) at 37°C.

4.3.1 **Passing the cells**

- Remove/aspirate the culture medium via a suction line in the hood connected to an external pump.
- Briefly rinse cells with 0.5 ml (1 ml is used for cell cultures grown on Ø100 mm dishes) of 0.25% trypsin (Gibco, Invitrogen) to remove all traces of serum that contains trypsin inhibitors.
- Add 0.5 ml (1 ml) of new trypsin to dish and gently rock/move the dish so that the solution covers the whole surface.
- Incubate the dish at 37°C, 5% CO_2 for 3-6 minutes until cells detach.
- Resuspend the cells in 4 ml (10 ml) of growth media.
- Transfer concrete amount of /Split the cell suspension to new dishes according to needs of the following experiment (lysate preparation, cell transfection, etc,).
- Place the culture dish in the 5% CO_2, 37°C incubator.

4.3.2 **Preparation of frozen stocks**

Culture should be approximately 80% confluence prior to preparation of frozen stocks in order to ensure the highest number of viable cell from reviving a frozen culture. It is possible to prepare two 1.5 ml cryogenic tubes of one large dish.

- Trypsinize cells as mentioned before (steps 1-4).
- Resuspend the cells in 5 ml of growth medium and transfer the cell suspension into 15 ml conical tube (Falcon).
- Centrifuge at 1000 rpm for 3 min (Eppendorf Centrifugen 5804R) at room temperature. Centrifugation at >1500 rpm results in cell death.
- Remove supernatant by gently aspirating it out without disturbing the cell pellet.
- Add appropriate volume, 1 ml – 1.5 ml of cryoprotectant/freezing solution.
- Resuspend pellet by gently pipeting up and down and transfer the cell suspension into cryogenic tubes. Make sure the tubes are capped tightly.

- Place tubes in the isopropanol-filled cryo 1°C container and transfer the container to -70°C O/N (24 hours the latest).
- The next day transfer frozen cells immediately to the liquid nitrogen for long-term storage.

Cryoprotectant solution 90%FBS (Sigma), 10% dimethylsulfoxid (DMSO)

4.3.3 **Reseeding the frozen cells**

We work with cells fast and keep them on ice when retrieved from the nitrogen container.

- Place the cryogenic tube in water bath (37°C) to thaw the cell suspension.
- Immediately after thawing, resuspend the cells in 5ml of preheated growth medium in a 15 ml conical tube.
- Centrifuge at 1000 rpm for 3 min at room temperature and remove supernatant by gently aspirating it out without disturbing the cell pellet.
- Add 4 ml of fresh growth medium, resuspend the pellet gently and seed the cells onto a small dish (after reseeding the cells from nitrogen storage resuspend the pellet in 10 ml of growth medium and seed it to large Ø100 mm dish).
- Check the cell culture under microscope.

4.3.4 **Transfection of mammalian cells**

For transfection cell culture should be app. 70 – 80% confluent. Transfection is performed with the use of jetPrime (Polyplus trasfection) reagent according to jetPRIME DNA transfection protocol showed in Figure 4.1. The transfection reagent does not interfere with serum so can be added to complete cell growth medium. The DNA used is purified on a NucleoSpin® Plasmid column and the concentration is measured with UV spectrophotometer (Schimazu UV 1650 PC).

In case the DNA used for transfection is coding fluorescently-tagged protein, cells should be checked by fluorescent microscope (Nikon Elipse TS 2000).

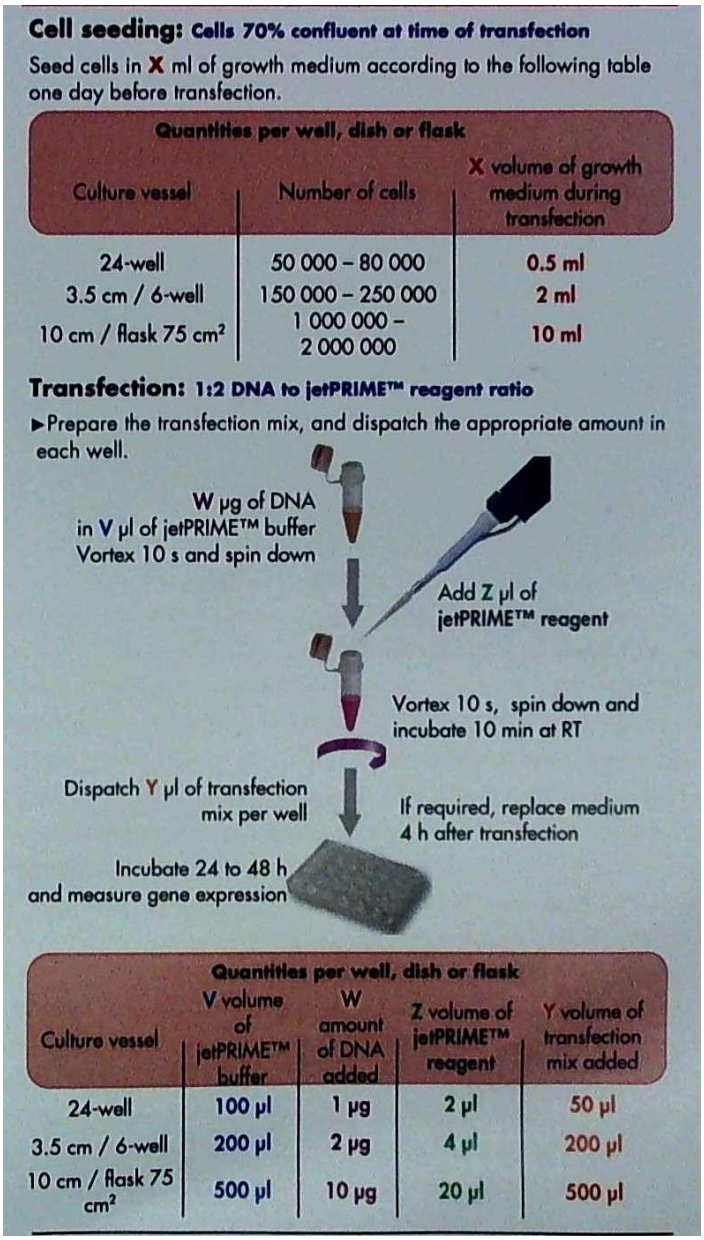


Figure 4.1 Schematic presentation of trasfection arrangement by jetPrime (Polyplus trasfection) reagents.

4.3.4.1 Immunohistochemical staining of mammalian cells

The cells are grown on microscope coverslips at least for 24 hours before staining. The coverslips are washed in HCl (helps the cells to stick to glass), rinse by ddH_2O and stored in pure ethanol.

- Singe the coverslips and place them into a 6-well tissue culture plate.

- Cover the slips with proteins of extracellular matrix. Dilute the stock solution of fibronectin (Serva) 1:100 in ddH_2O and pipet 100 µl per a coverslip. Dry the covered slips at safe place in flow box.
- Seed the cells on a coverslip according to needs of the following experiment; i.e. dilute the cell suspension so that the confluence at the time of fixing the cells/next day is app. 50%. Supply the cell culture with 2 ml of growth medium.
- Aspirate the medium and wash the cells twice with 1xPBS. This step and all the following steps are performed in non-sterile conditions.
- Fix cells with 4% of paraformaldehyde in 1xPBS for 10 – 15 minutes.
- Wash the coverslips three times with 1xPBS, 10 minutes for the first wash.
- Permeabilize cells with 0.3% Tritone X-100 in 1xPBS for 10 – 15 minutes.
- Wash the coverslips three times with 1xPBS, 10 min the first wash.
- Set up humidified chamber (large glass plate with a lid and water-saturated tissue in it; bottom lined with parafilm). Place coverslips into the humidified chamber with cells facing up. Block the cells by pipeting 200 µl of 3% BSA in PBS onto a coverslip for 30 – 60 minutes.
- Dilute primary antibody 1:50 in 3% BSA. Drop 50 µl of the solution onto the parafilm and carefully place a coverslip upside down, cells facing down. Incubate the cells in humidified chamber for 3 hours.
- Wash the coverslips three times with 1xPBS, 10 min the first wash.
- Dilute secondary antibody 1:500 in 3% BSA. Drop 100 µl of the solution onto the parafilm and place a coverslip upside down. Incubate the cells in humidified chamber for 1 hour.
- Wash the coverslips three times with 1xPBS, 10 min the first wash.
- Dilute phalloidin stock solution 1:70 in 3% BSA. Drop 70 µl of the solution onto the parafilm and place a coverslip upside down. Incubate the cells in humidified chamber for 15 minutes.
- Wash the coverslips three times with 1xPBS, 10 min the first wash.
- Rinse the coverslips in distilled water to discard the salt from PBS. Remove the remaining water and place the coverslips upside down onto a 8 – 10 µl drop of mounting medium. The slips can be stored in fridge, 4°C until analyzed by fluorescent confocal microscope (Leica TCS SP2).

DY-405-phalloidin (Dyomics)

Mounting medium	12% Mowiol, 30% Glycerol, 20mM Tris, 0.95 g/l sodiumazid

4.3.5 **Preparation of whole cell extract from mammalian cells**

Cell lysates are prepared on ice in non-sterile conditions. Cell culture should be 80 - 100% confluent and 0,4 ml of lysis buffer is used for small and 1 ml for large dishes.

- Transfer the dish of cells on ice and cool it down for 5 minutes.
- Aspirate the medium and rinse the dish twice with cold 1xPBS. Aspirate the whole PBS.
- Add 0.4 ml (1 ml) of RIPA or Hepes/Nonidet buffer containing protease inhibitors and incubate for 12-15 minutes on a rocker (Biosan, MR-1) in cold room.
- Scrape cells to one area (at the edge of the dish) with plastic cell scraper and collect the suspension into a pre-cooled 1.5 ml microfuge tube.
- Pass cell lysates through 21G needle 8 – 10 times to homogenize the solution
- Centrifuge the suspension at 12 000 rpm and 4°C for 20 minutes (Eppendorf Centrifuge 5417R).
- Transfer the supernatant gently to a new 1.5 ml microfuge tube. Remove 25 µl of the lysate for determining the protein concentration.
- The lysates can be stored in -20°C up to 6 months.

RIPA lysate	0.15 NaCl; 50mM Tris –HCl (pH 7,4); 1% Nonidet P-40; 0.1% SDS; 1% sodium eoxycholate; 5mM EDTA; 50mM NaF
Nonidet	20mM Hepes; 100mM KCl; 0.5% Nonidet P-40; 1mM DTT

Stock solution of protease inhibitors (1000x):

0.5 mg/ml leupeptin
0.5 mg/ml aprotinin
12.5 mg/ml pefablock

Phosphatase inhibitors Mix (Serva, Germany) (100x)

Non-ionic detergents such as Nonidet P-40 (NP-40) are less harsh then ionic detergents that contain SDS or deoxycholate. NP-40 is non-denaturating and used for antigens that are detergent soluble and can be recognized in native form by an antibody. RIPA (RadioImmunoprecipitation Assay) buffer is more denaturating, useful for nuclear membrane disruption, but can denaturate kinases.

4.3.5.1 Measurement of protein concentration in cell lysate – Folin´s method

Protein concentration is determined by colorimetric assay with D_c Protein Assay kit (Bio – Rad, USA).

- Prepare the serial dilution with bovine serum albumin (BSA) as following:

BSA 2mg/ml (µl)	Buffer RIPA/Nonidet (µl)	Final concentration BSA (µg/µl)
0	50	0
5	45	200
10	40	400
20	30	800
37,5	12,5	1500

- Add 25 µl of RIPA/Nonidet lysis buffer to 25 µl of the discarded cell lysate suspension. Keep on ice.
- Prepare solution A´ by adding add 20 µl of reagent S to 1 ml of the A reagent. Pipet 125 µl of solution A´ to 25 µl of the standard BSA dilution or 25 µl of sample solution prepared in previous step.
- Pipet 1 ml of B solution to each of the prepared protein solution. Incubate in dark for at least 15 minutes.
- Measure the absorbance of the sample at 750 nm wave length (Schimadzu UV 1650 PC).
- Create calibration curve from data of BSA dilution serial which used thereafter for determining of the concentration of sample solution.

4.4 Proteomics

4.4.1 **Purification of GST fused proteins**

We perform all the experiments on ice since the step when lysing the bacteria cells! We work with GST-sepharose 4B all the time gently (centrifugation at max 4400 rpm, 4°C, votex gently).

- Inoculate 20 ml of YTG medium with Ampicillin (100 µg/ml) with *E. coli* cells BL21 harboring pGEX plasmids with variants of p130Cas domains (WT – SH3 wild type, E – SH3 domain mutant Y12E, F – SH3 domain mutant Y12F, Hs – short variant of CAS CCH domain, Hl – long variant of CAS CCH domain, Gst – empty GST vector). Shake at 180 rpm and 37°C (O/N)
- Check the optical density (OD) on a spectrophotometer next day. OD_{600} should be 1.4 – 2.4. Pure YTG is used as blank.
- Inoculate 50 ml of new YTG+amp with grown cell culture so that the final OD_{600} is app. 0.2. Shake at 180 rpm at 37°C until the OD_{600} reaches app. 0.5 (usually 1hour).
- Remove 1 ml of cell culture that will be used as a negative control before induction of GST-recombinant protein expression. Centrifuge (Hettich MIKRO 20) the samples at 11 000 rpm for 1 minute, wash pellet twice with cold LB2 and resuspend the pellet in 100 µl of LB1. Add 20 µl of 6x Laemmli+DTT (1M diluted 20x) (sample –IPTG)*.
- Transfer the culture into 30°C and incubate at 180 rpm for 15 minutes.
- Add IPTG to final concentration 1mM to cell culture and incubate at 180 rpm and 30°C for 1.5 hour. Check OD_{600}. It should be 1 – 2.3.
- Remove 1 ml of cell culture that will be used as a positive control after induction of Gst-domain recombinant protein expression. Centrifuge (Hettich MIKRO 20) the samples at 11 000 rpm for 1 minute, wash pellet twice with cold LB2 and resuspend the pellet in 100 µl of LB1. Add 20 µl of 6x Laemmli+DTT* (sample +IPTG).
- Cool down the culture on ice and transfer it to 50 ml conical tube. Pre-cool the centrifuge (Jouan 22i) meanwhile.
- Centrifuge the cells at 4400 rpm and 4°C for 15 minutes.

- Remove supernatant and resuspend sediment in 5 ml of pre-cooled LB2 and supply 20 ml. Centrifuge the cells at 4400 rpm and 4°C for 15 minutes. Repeat this step twice to wash the cells.
- Resuspend the pellet in 2 ml of pre-cooled LB1 buffer with protease inhibitors.
- Lysate the cells with French press (Thermo Electron). Adjust the pressure to 600 atm ($6.08x10^7$ Pa) and apply the pressure.
- Transfer the lysate directly to 100 µl of 20% Triton X-100 so that its final concentration is 1%. Add 1000x protease inhibitors immediately. Incubate the lysate for 25 minutes in cold room. The samples can be also stored at -20°C O/N at this step.
- Centrifuge (Hettich MIKRO 20) lysate at 13 000 rpm at 4°C for 20 minutes. Transfer the supernatant to new 1.5 microfuge tubes. This will be used for isolation of GST-recombinant proteins.
- Wash GST-sepharose™ 4B (GE Healthcare) three times with Hepes (centrifuge at 2400 – 4400 rpm, 4°C, 1 min → discard supernatant →add new Hepes) and dilute it in LB1 so that the final concentration is 50%. Add 120 µl of washed 50% GST-sepharose 4B to each of the lysate.
- Incubate the sepharose beads with the lysate on a laboratory rotator for 1 hour in cold room. Try to avoid bubbles in lysate.
- Discard supernatant and wash GST-domains bound to sepharose beads three times with LB1 (centrifuge at 2400 – 4400 rpm, 4°C, 1 min → discard supernatant →add new LB1). Add 60 µl of LB1+pI so that the final concentration of the beads is 50%.
- Remove 5 µl of the suspension that will be used as a control for GST-domain purification. Add 20 µl of 2xLammli+DTT* (sample GST-domain).
- In case of repeated use prepare stock solution of isolated GST-domains by adding glycerol so that its final concentration is 10% and store it in -20°C.
- *To check the production and purity of isolated GST-domains boil three samples (-IPTG, + IPTG, GST-domain) at 95°C for 10 minutes and run SDS-PAGE (Figure 5.2).

IPTG 238 mg/ml

LB1uffer 59mM Hepes, 100mM NaCl, pH 7.4

LB2 buffer 10x diluted LB1

4.4.2 **Pull-down assay**

The pull down assay is an in vitro method used to determine a physical interaction between two proteins. Pull-down assays are useful either for confirming the existence of a protein-protein interaction predicted or as an initial screening assay for identifying previously unknown interaction (Schematic representation of protein purification and a pull down assay is shown in Figure 4.2).

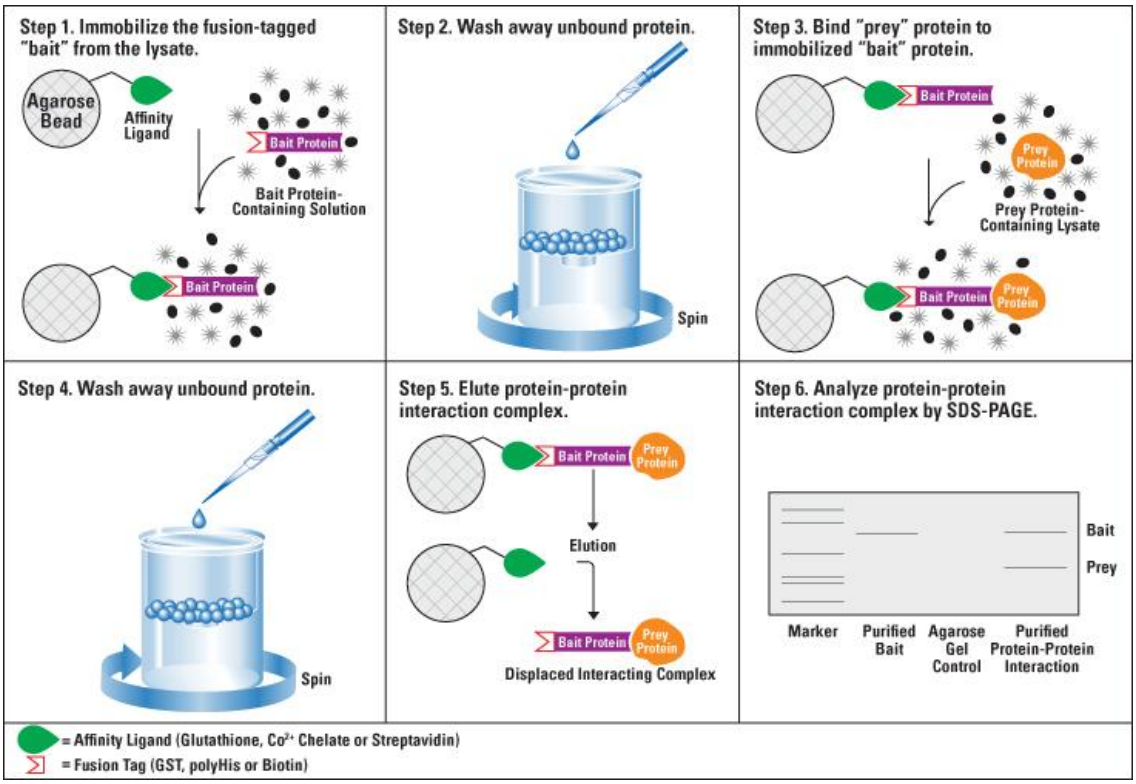


Figure 4.2 Schematic representation of a pull down assay. Affinity-purified proteins are incubated with a cell lysate. The binding partners are immobilized on agarose beads together with the purified protein. Unspecific interactions are washed away by washing steps. The interacting proteins are separated on SDS-PAGE (adopted Thermo SCIENTIFIC web-page).

We work with isolated domains (GST-domain variants bound to sepharose beads) on ice during the whole experiment. The sepharose beads should never get dry.

- To remove glycerol wash domains three times with LB1+pI (centrifuge at 2400 – 4400 rpm, 4°C, 1 min → discard supernatant →add new LB1) and add LB1 to final concentration 50%.
- Transfer 20 µl of 50% sepharose suspension to a new 1.5 microfuge tube.

- Immediately apply 1 ml of Hepes/Nonidet protein lysate (protein concentration app. 1 mg). Remove 50 µl of lysate, add 10 µl of 6xLaemmli+DTT (20x) and boil sample at 95 °C for 10 minutes. The sample is used as a positive control.
- Incubate lysate with sepharose at 4°C for 2 hours on a laboratory rotator.
- Wash the sepharose beads three times with LB1 as described before.
- Centrifuge the sepharose suspension and remove supernatant. Aspirate the remaining LB1 with 21G needle.
- Immediately add 30 µl 2xLaemmli+DTT to sepharose beads.
- Eluate the bound proteins by boiling samples at 95 °C for 10 minutes.
- Separate proteins by SDS-PAGE and continue with either Western blot experiment or preparation of samples for mass spectrometry analysis.

4.4.3 **Sample preparation for mass spectrometry**

The work should be performed carefully with the use of laboratory gloves to decrease the possibility of keratin contamination while handling samples. Use 0.75 mm PAGE gels for separation of proteins.

- After separation of proteins by SDS-PAGE stain the gel with fresh Coomassie blue to visualize the protein bands.
- Cut a protein band from the gel with sharp scalpel (Figure 4.3) and re-cut it to app. 1 mm pieces. Avoid cutting bands that are visualized also in negative control (proteins interacting with empty GST).
- Transfer the gel pieces into 0.5 microfuge tube and add 90 µl of H_2O used for MS.
- To wash out the Coomassie stain and SDS aspirate water carefully and add 1 µl of DTT (1M) and 90 µl of 50mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN). Vortex strongly for 10 s and incubate for one minute at 60°C in heating block. Repeat this step until the gel is destained.
- Aspirate carefully the whole volume of ABC/ACN.
- For protein alkylation add 50 µl of iodoacetamide (IAA) in ABC/ACN, vortex strongly and incubate at 60°C for 20 minutes. IAA is poisonous.
- For protein dehydration aspirate IAA and add 90 µl of ACN, vortex strongly. The gel should become shrinked and white.

- Aspirate ACN and add 90 µl of H_2O for protein rehydration, vortex strongly.
- Aspirate water and add 90 µl of ACN, vortex strongly.
- Discard the whole volume of ACN and dry the gel (keep the microfuge tube at distant/safe place with open lid) for app. 10 – 20 minutes. The whole gel should become white.
- Pipet 1 µl of trypsin directly onto the gel.
- Supply with 8 -12 µl of ABC (volume according to size of the shrinked gel).
- Incubate in 37°C O/N.
- The next day take samples to MS analysis or add trifluor acid of same volume as ABC. Sonicate for 7 minutes and store in -20°C.

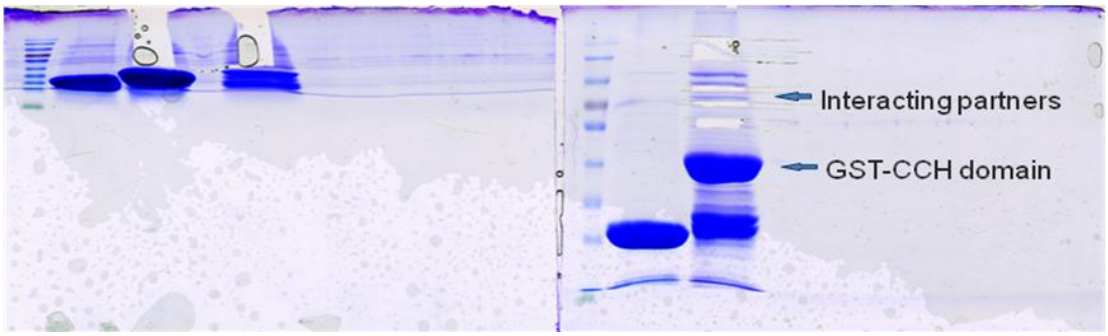


Figure 4.3 Presentation of gels used for MS analysis. The interacting partners (at the top part of the gel) of GST recombinant proteins (large band in the rather bottom part of gel) are separated by SDS-PAGE electrophoresis. The gel is stained with fresh Coomassie stain and destained with destaining solution. The interacting proteins are cut off the gel and samples are made of them for the MS analysis. Another option is to run the gel only app.1cm (left gel) and cut out the proteins non-specifically. In this case the samples prepared are analyzed by tandem MALDI/TOF-TOF.

4.4.4 **Immunoprecipitation**

Immunoprecipitation (IP) is a technique that uses antibodies specific to a protein to remove those proteins from solution. The antibody-protein complexes are isolated out of solution by binding the Protein A immobilized on sepharose beads. In our case, we confirm the specificity of new identified interacting partners of CAS anchoring domains by this method (new interacting partner is co-precipitated with CAS).

Work with sepharose beads gently and avoid getting them dry. To prepare protein lysates Nonidet P-40 is preferred as it is a non-ionic detergent and will not denaturate proteins or disrupt protein:protein interactions. Polyclonal antibodies should be used for

IP. In case the precipitated or co-precipitated protein has high affinity for Protein A, preclearing step is done.

- Prepare whole cell extract from desired cell line with the use of Nonidet P-40 lysis buffer as described before (chapter 4.3.5). Use approximately 1 mg of total protein for IP.
- Pre-clear the protein lysate with 20 µl of 50% Protein A-sepharose (GE healthcare) solution by incubating it on lab rotator at room temperature for 1 hour. Protein A-sepharose solution is stored in ethanol that is why it has to be washed three times with LB1 buffer before use (2200 rpm, 4°C, 2 min, Hettich MIKRO 20).
- Spin down the sepharose beads and use pre-cleared lysate for the following steps.
- Add 1 – 2 µg of polyclonal antibody to the lysate and incubate in on laboratory rotator in cold room O/N. Avoid bubbles.
- Collect/pull down the desired protein complexes by an additional incubation with 30 µl of 50% Protein-A sepharose.
- Wash protein complexes bound to sepharose beads three times with the LB1 buffer (2200 rpm, 4°C, 2 min, Hettich MIKRO 20).
- Aspirate the remaining LB1 buffer with the use of 21G needle and immediately! Add 30 µl of 2xLaemmli sample buffer with DTT.
- Elute proteins by boiling the samples at 95°C for 10 minutes and at 100°C for 2 min. Shake gently 2 – 3 times.
- Store the samples at -20°C or use for SDS-PAGE and immunoblotting.

4.5 Bioinformatics

The bioinformatic analysis was used to analyze tyrosine phsphorylation within SH3 domains. Only publicly available resources were used.

4.5.1 **A domain phosphorylation data collection**

Phosphosite Plus *(PSP) is an online system biology resource that studies protein post-translational modifications. It is re-engineered from previous PhosphoSite (Hornbeck et al., 2004) and stores phosphosites from low-throughput (LTP), high-throughput (HTP) and cell signalling technology (CST). Up to 10 000 journal articles since 2001 have*

been manually curated by expert scientist to collect the data. HTP sites, discovered with the use of mass spectrometry (MS2), come not only from published literature but also from previously unpublished data generated at CST. PhosphoSite Plus provides information about the phosphorylated residue and its surrounding sequence, orthologue sites in other species, location of the site within known domains and relevant literature references (Hornbeck et al., 2004). This makes PSP one of the most comprehensive databases of post-translational modifications.

- To search for phosphorylated SH3 domains a database of protein phosphorylation sites such as PSP should be used.
- With Site search option in PSP retrieve a list of modified sites within SH3_1 and SH3_2 domains.
- For the further analysis use only phosphotyrosine (pY), phosphoserine (pS) and phosphothreonine (pT) hits.
- Calculate the ratio of tyrosine to all phosphorylations.

The ratio of tyrosine to all phosphorylations in other adaptor domains (SH2, PH, PDZ, WW, PTB, EH, PX) can be determined with the same approach. To avoid redundancy use only human domains for the calculation.

- Perform normalization to the percentage of tyrosines in adaptor domains (as defined in Pfam) versus percentage of these aa in human proteom (as defined in Uniprot) (Consortium, 2012; Finn et a., 2010). We first count the ratio of phosphorylated tyrosines to percentage of tyrosines in human proteome and in the domains and then count the prevalence of tyrosine phosphorylation in the domains compared to human proteome.

4.5.2 **Abundance of phophotyrosine sites in a domain and motif identification**

- Check the occurrence of each phosphorylation site within SH3 domain by comparing it with the Simple Modular Architecture Research Tool (SMART)-defined SH3 domain sequences. Exclude all phosphorylation hits beside SMART-defined SH3 domains.

- From SMART domain definition program (Letunic et al., 2009; Schultz et al., 1998) retrieve all SH3 domain sequences that according to PSP contain tyrosine phosphorylation.
- Align the SH3 domain sequences with the use of Multiple sequence alignment (MSA) tool, ClustalW (Thompson et al., 1994). FASTA format of sequences is necessary. Exclude orthologue and paralogue sequences before creating the MSA for the sake of clarity of the alignment.
- Use MSA of tyrosine phosphorylated SH3 domains for further analysis.
- With the use of WebLogo application (Crooks et al., 2004) http://weblogo.berkeley.edu/logo.cgi create sequence logo around the most frequently phosphorylated tyrosine positions.

4.5.3 **ALYDY/F motif search in human SH3 domains**

- Retrieve all human SH3 domain sequences from SMART and SH3_1 from Pfam database (Finn et al., 2010).
- Use simple text search to locate ALYDY/F motif in SH3 domain sequences with respect to localization of the motif within each domain sequence.

5 Results

Mechanosensory role of p130Cas in focal adhesions is highly dependent on anchoring the protein to the cell membrane at these sites (Sawada et al., 2006). According to previous studies the focal adhesion-targeting domains of CAS are SH3 domain at the N-terminus and CCH-domain at the C-terminus (Donato et al., 2010; Harte et al., 2000; Nakamoto et al., 1997). Very little is, however, known about the nature of binding partners of these two domains that are responsible for CAS anchorage to the cell membrane. The main aim of this thesis was to find new interacting partners of CAS anchorage domains which would contribute to better understanding of the mechanosensory role of CAS in FA. We followed standard proteomic approach in order to answer this question. We affinity-purified the two FA targeting domains of CAS, the SH3 domain and the CCH domain through a GST tag. Next, we performed pull-down assays and separated the interacting proteins by SDS-PAGE. The in-gel digest of proteins and mass spectrometry analysis revealed new interacting partners of CAS of which some are very probably responsible for targeting CAS to FA. Meanwhile, we also indicated the possible role of CAS CCH domain in podosomes. Results based on phosphomimiking mutation of SH3 domain at position Y12 led to bioinformatic analysis that focused on tyrosine phosphorylation within the set of all SH3 domains.

5.1 Preparation and purification of CAS SH3 and CCH anchoring domains

5.1.1 **Preparation of GST expression vectors**

Three variants of GST-SH3 construct were prepared previously by Dr. Rösel: wild type GST-SH3_WT (Supporting data S5.1), GST-SH3_E harboring a phosphomimiking mutation Y12E, GST-SH3_F harboring a non-phosphorylatable mutationY12F. Original CAS cDNA comes from mouse. We have used these constructs through this study.

Two variants of GST-fused CAS CCH domains were used in our experiments: long variant GST-Hl contains the C-terminal 181 amino acids of p130Cas (693 – 874 aa according to mouse numbering) and short variant GST-Hs covering last 133 amino acids (741-874aa). The cDNA CAS-CCH fragments were amplified by PCR using N-

terminal forward primers 5′GGATCCATGCGGCAGGGAAAGGGC 3′ or 5′GGATCCGGGCCTTCAGACCGACAG 3′ respectively and C-terminal reverse complementary primer 5′CCGAATTCTCAGGCAGCAGCTAGC 3′. pIRES-puro-CAS plasmid was used as a template DNA (Janostiak et al., 2011). The PCR produced DNA fragments were cloned into pCR2.1.TOPO vector with the use of TOPO TA kit (Invitrogen). *E.coli* DH5α competent cells were transformed with the ligation mixture and were streaked on selective plates containing ampicilline. Two colonies of each variant were picked and the plasmid DNA was taken for sequencing. The CAS CCH cDNA (for both variants) insert was recovered after digestion with BamHI and EcoRI, purified from agarose gel and ligated in the same sites of the expression plasmid pGEX:Src-SH3 new expression pGEX-CAS-Hl and pGEX-CAS-Hs plasmids were created (Figure 5.1, Supporting data S5.2). After expression and isolation of GST-CCH domains, the CCH domain was verified by MS analysis.

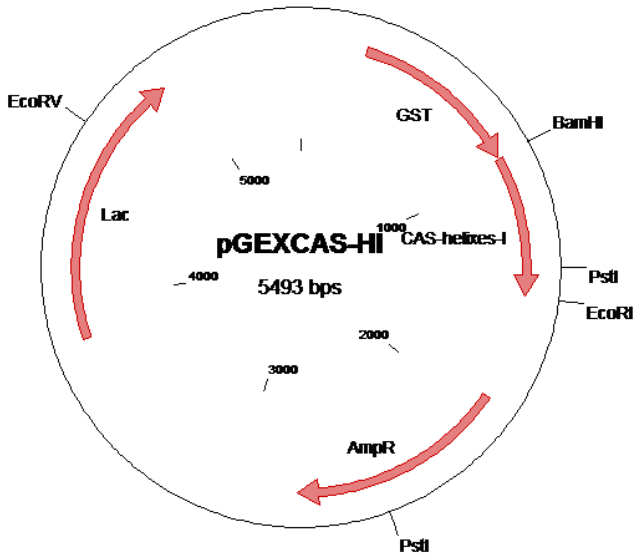


Figure 5.1 A schematic map of bacterial expression plasmids pGEX-CAS-Hl showing restriction sites and domain structure. Size of vector fragment is 4938bp and size of insert is 555bp (411bp for short variant, construct shown in supporting data S5.2). Restriction enzymes used for plasmid preparation were BamH1 and EcoR1.

5.1.2 **Optimizing conditions for expression and purification of GST-CCH recombinant proteins**

E.coli BL21 bacterial cells were transformed with expression plasmids described above. The cells were used for expression and purification of GST ("empty" **ØGST** was used in later experiments as a negative control, **26 kDa**) and GST-fused CAS domains:

GST-SH3_WT (wild type form of CAS SH3 domain fused with GST), ~**32kDa**

GST-SH3_E (GST fused CAS-SH3 domain harboring a phosphomimiking mutation at position Y12, Y12E),

GST-SH3_F (GST fused CAS-SH3 domain harboring a non-phosphorylatable mutation at position Y12, Y12F)

GST-Hl (GST fused CAS-CCH domain, long form, C-terminal 181 amino acids), ~**46 kDa**

GST-Hs (GST fused CAS-CCH domain, short form, C-terminal 133aa), ~**40kDa**

The expression of recombinant proteins was induced in BL21 cells by adding 1mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Figure 5.2, +IPTG). Thereafter, we tried to isolate as much as possible of an expressed recombinant protein. GST-fused domains were isolated by affinity purification via strong binding to gluthation immobilized on sepharose beads (GluthationSepharose^{TM} 4B, GE Healthcare) (Figure 5.2, lane with domain description). Thus, the efficiency of the protein expression and purification was always verified by SDS-PAGE electrophoresis (Figure 5.2).As we can see the original protocol (diploma thesis of Mgr. Martin Sztacho, chapter 3.16) was satisfying for isolation of GST and GST-fused SH3 domains. However, the results were very poor in case of CAS CCH domains (arrows at the right gel).

There were two possibilities how to increase the efficiency of GST-CCH proteins isolation:

1. To optimize the conditions of protein expression in bacteria
2. To optimize the conditions of protein isolation/purification from bacterial lysate

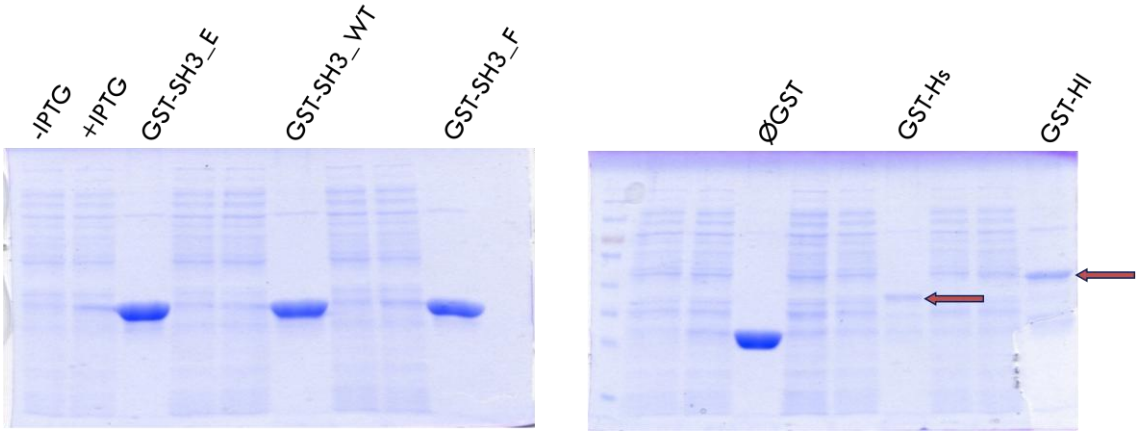


Figure 5.2 Illustration of CAS anchoring domains expression and purification. The first of three lines is always a sample of bacterial cell culture (transformed with the concrete pGEX expression plasmid) before addition of IPTG. Second line represent sample of bacteria culture app. 1.5 hour after IPTG induction. The third line is showing purified GST recombinant protein: ØGST („empty" GST), GST-SH3 (WT - wild type CAS SH3 domain, E - Y12E substituted CAS SH3 domain, F – Y12F substituted CAS SH3 domain), GST-CCH (Hs – Helix short, C-terminal 133 amino-acid-region of CAS, Hl – Helix long,181aa C-terminal region of CAS).

1. When optimizing the expression condition we focused on change of four parameters:

- Optical density, OD_{600} of bacterial culture at the time of IPTG induction (0.55 or 1)
- The temperature of bacteria culturing after IPTG induction (37°C or 30°C)
- Concentration of IPTG for induction of protein expression (1mM or 0,5mM)
- Length of induction (60-240 minutes)

By this we created several different conditions for GST-fused protein expression in BL21 bacterial cells:

OD_{600}	**[IPTG]**	**°C**	**60´**	**90´**	**120´**	**180´**	**240´**	**culture**
0,55	1mM	37	*	*	*	*		1
0,55	1mM	30		*	*	*	*	2
0,55	0,5mM	37		*	*	*	*	3
1	1mM	37	*	*	*	*		4
1	1mM	30	*	*	*	*	*	5
1	0,5mM	37	*	*	*	*	*	6

We verified the expression efficiency by SDS-PAGE. From picture below (Figure 5.3) it seems that the best conditions for GST-Hl domain expression are: Grow the cell

culture approximately to OD_{600}=0.5. Transfer the culture to 30°C and after few minutes (10-15min) induce the protein expression with 1mM IPTG. Incubate the cell culture for 4 hours (Figure 5.3, conditions (2)). With this approach we reached at least an eight-fold increase in GST-Hl domain expression.

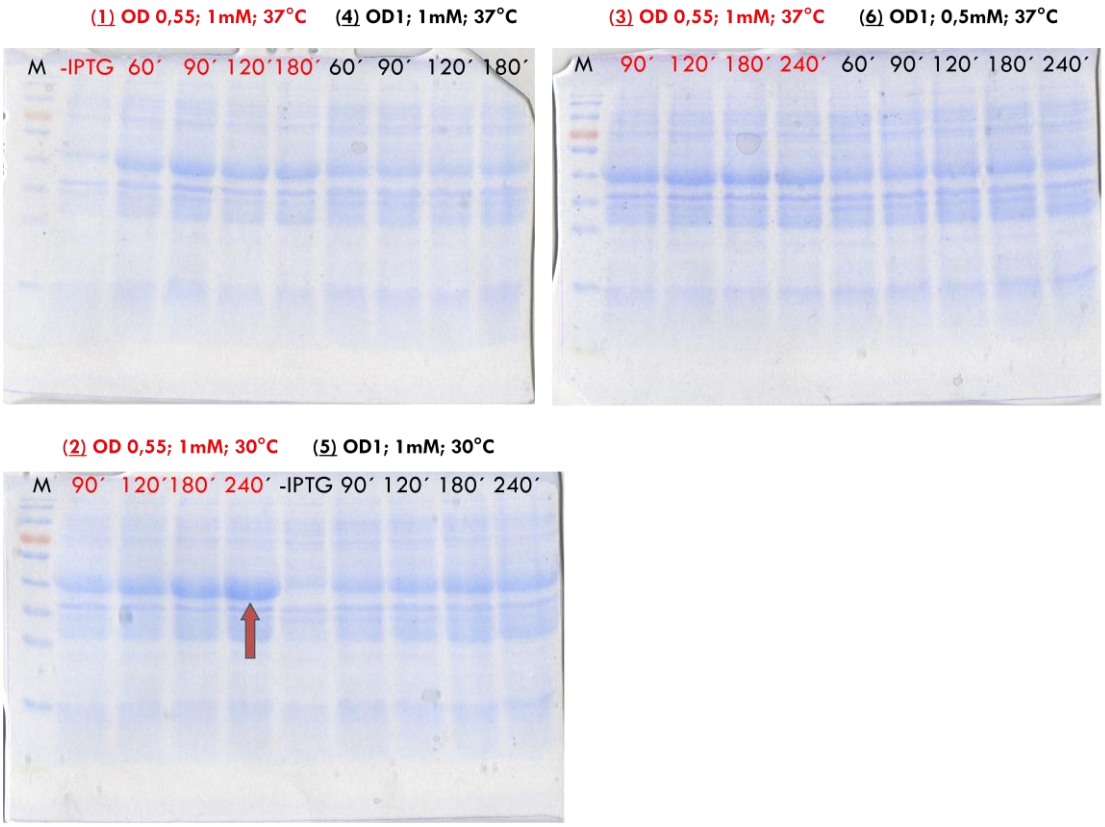


Figure 5.3 SDS-PAGE electrophoresis of samples used for optimizing the expression conditions of GST-Hl recombinant protein. BL21 cells transformed with pGEX-CAS-Hl were grown under different conditions (described above the gel) e.i.: (1) The culture was grown to OD 0.55, the expression of GST recombinant protein was induced by 1mM IPTG and subsequently grown at 37°C for 60, 90, 120, 180 minutes. Always one ml of cell culture was used for analysis of the condition (centrifuged at 11 000 x g for 1 min, pellet washed with LB2 and resuspended in 100 µl of LB1, 20 µl of 6xLaemmli+DTT sample buffer was added and the sample was heated to 95°C for 10 minutes). The arrow shows the best conditions for GST-Hl expression in BL21 bacterial cells.

Another problem occurred, when trying to purify the CCH domains from bacterial lysate. Even we increased the expression level, it does not correspond to the level of purification efficiency. This was true especially for GST-Hs purification (Supporting data S5.3A). It seemed that the OD_{600} of cell culture used for protein extraction negatively correlated with the yield of purified domains. This could mean that the CCH domains are highly hydrophobic and aggregate strongly forming inclusion bodies. Thus when expressing GST-domains we followed the previous conditions (described above),

however, with one exception: We grew the bacterial cell culture after IPTG induction up to OD_{600}=2! Final protocol used for purification of GST-fused CAS anchoring domains is summarized in chapter 4.4.1. With this protocol we were able to purify "empty" GST and GST-SH3 domains with a 10-fold increase (~ 13mg/ml) when compared to the early purification experiments.

1. When extracting and purifying CAS CCH domain from bacterial cells, additional conditions were changed:

   - The cells were lysed in 200 – 400mM NaCl LB1 buffer (200mM preferably).
   - Triton X-100 was added to final concentration 2% and the cell suspension (after French press) was resuspended as much as possible!
   - The lysate (supernatant after centrifugation) was incubated with sepharose beads overnight at 4°C (or 4 hours at least).
   - Avoiding addition of glycerol (10%) for storage of the isolated domains helped a lot as well. That meant that the isolated domains were used immediately for pull-down experiments.

Finally the concentration of GST-Hl domain was 3.5 mg/ml. For pull-down experiments we tried to equal concentrations of isolated domains.
The problems with purification of short form of CAS-CCH domain persisted (Supproting data S5.3B). That is the reason why we did most of the experiments using the long form CAS CCH domain, GST-Hl.

5.2 Identification of new interacting partners of p130Cas focal adhesion targeting domains

After isolation of CAS SH3 and mainly CCH domain in sufficient amounts we were able to incubate the domains with cell lysates and pull down the interacting partners of the domains from lysate. These were subsequently determined by mass spectrometry.
Hela cells were grown in RPMI medium to confluence and were extracted with 0.5% NP-40 lysis buffer. For each pull-down experiment a cell lysate of protein amount app. 1 mg and 30 µl of 50% sepharose-domain suspension with the lowest concentration were used. Volume of the other domains was added so that the domains concentration

was the same in one experiment. Sepharose-immobilized domains were incubated with protein lysate to pull-down interacting partners from it. The beads were washed extensively, boiled in Laemmli sample buffer and proteins were separated by SDS-PAGE electrophoresis. Gels were stained with Coomassie Blue, and the identified bands were excised, processed and analyzed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectroscopy. When excising bands of gel non-specifically (Figure 4.3, left gel) the samples were analyzed by tandem TOF/TOF MS (Applied Biosystems). The MS analysis was performed at the Service laboratory of mass spectrometry of Charles University in Prague by Mgr. PetrJedelsky. For interpretation of peptide fragments MASCOT (MATRIX SCIENCE) server was used. A total of ~ 100 samples were taken for MS analysis during this study. By this approach we were able to identify several interacting proteins of CAS SH3 and CAS CCH domain (Table 5.1). Some of the identified proteins, PTK2 and CD2-AP were previously described to interact with SH3 domain of CAS (Kirsch et al., 1999; Polte and Hanks, 1995). These proteins therefore served as a positive control of the method used for identification of interacting partners for CAS SH3 and CCH domain for us.

Of newly discovered CAS SH3 domain binding proteins vinculin was the most significant result. That is the reason why we paid the most attention to the interaction CAS/vinculin which will be described more specifically later (chapter 5.4).

The MS-analysis suggests also the interaction between CAS SH3 domain and Neural Wiskott-Aldrich syndrome protein (N-WASP), SH2 domain-containing protein 4A (SH2D4A) and WD repeat-containing protein 44 (WDR44). We did not study these newly identified proteins in more details.

α-Tubulin, β-5-Tubulin and BCAR1 were identified as CAS CCH domain binding proteins. We tried to verify these interactions.

We had several possibilities how to confirm the new interactions.

- Pull-down experiment of GST-domain with NP-40 lysate of Hela cells that was followed by immunodetection of newly identified protein.

- After PD experiment the specificity of the interaction was confirmed by immunoprecipitation of full-length CAS from Hela cells and immunoblotting of precipitated CAS and co-precipitated interacting partner.

- Immunofluorescent experiment with the use of MEF cells (or derived) helped to determine the role of the interacting protein in localization of CCH to focal adhesion.

CAS-domain	Interacting protein	ID number
SH3_F	Vinculin	Q64727
	Neural Wiskott-Aldrich syndrome protein	Q91YD9
	SH2 domain-containing protein 4A	Q9ES52
	WD repeat-containing protein 44	Q6NVE8
	Tyrosine-protein phosphatase non-receptor type 11(P35235), Adaptor protein HOF1 (Q05080), Ras-GTPase-activating protein SH3-domain-binding protein (Q9UN86)	
SH3_WT	PTK2	Q8IYN9
	CD2-AP	Q9JLQ0
	Vinculin	Q64727
	BCAR1 (Q61140), BAT 2 (P48634)	
SH3_E	Macrophin (Q9UPN3)	
Cas_Hl	α-tubulin, β-5-tubulin	P05213 P68372
	BCAR1	F5GXA2
	CEP152 (O94986-2)	

Table 5.1 A list of mass spectrometry identified interacting partners of CAS SH3 or CCH domain. Cas-domain column shows CAS anchoring domains that were used for pull-down experiment. The second slope shows MS identified interacting proteins of the concrete domain. Proteins of light color were hits with low score. We searched for UniProt codes according to the GI number (if provided from MS service laboratory) or according to protein name.

5.2.1 **CAS CCH domain interacting partners**

5.2.1.1 Preparation of GFP fused CAS CCH domains

CCH domain GFP (green fluorescence protein) construct was generated of insert that was previously purified for the pGEX plasmid preparation (chapter 5.1.1). cDNA for CAS CCH domain (both variants Hl-181aa, Hs-133aa) was subcloned into GFP-CAS (chapter 3.7.3) plasmid that was digested with BglII and EcoRI restriction endonucleases (MBI Fermentas), so that the CAS CCH domain was expressed with an N-terminal Green Fluorescent Protein (GFP) tag (Figure 5.4, Supporting data S5.4). The newly prepared GFP plasmids containing CCH variants (GFP-CAS-Hl and GFP-CAS-Hs) were verified by re-digestion (Supporting data S5.5). Expression of the GFP tagged

protein in MEF cells was achieved by transient transfection with JET prime reagents (Polyplus trasfection). Localization of GFP-Hl and GFP-Hs in cells was detected by fluorescent confocal scan microscope (Leica TCS SP2) as described in later chapters (5.2.1.3 and 5.3)

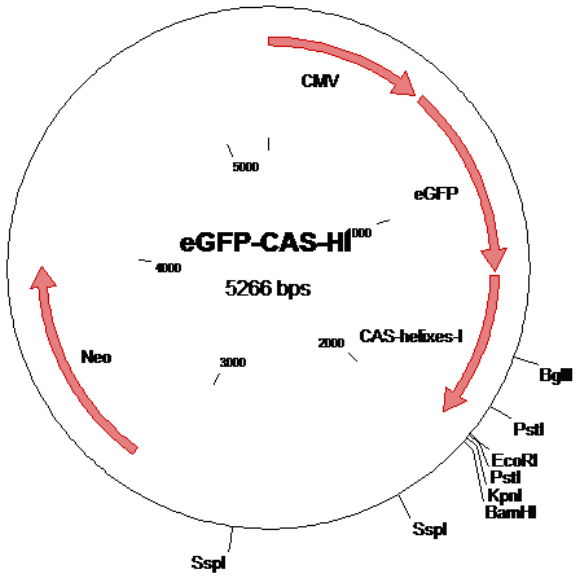


Figure 5.4 A schematic map of bacterial expression plasmids pEGFP-CAS-Hl showing restriction sites and domain structure. Size of vector fragment is 4711bp and size of insert is 555bp (411bp for short variant, construct shown in supporting data S5.4). Restriction enzymes used for plasmid preparation were BglII and EcoRI.

5.2.1.2 CAS CCH domain interaction with tubulin and CAS

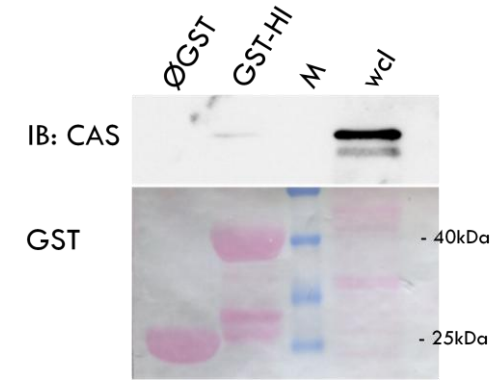
Two proteins were identified to be interacting with CCH domain of CAS by mass spectrometry, tubulin and BCAR1, a human homolog of p130Cas. The interaction CAS-CCH/tubulin could be confirmed neither by PD experiment nor co-localization of GFP-CAS-Hl and tubulin in MEF cells was detected (data not shown).Thus we did not confirm the interaction between CAS CCH domain and tubulin that was suggested by MS analysis

Identification of BCAR1, a human homologue of p130Cas as the interactor of CAS-CCH led to an assumption that CAS most probably homodimerize through the CCH region. This notion was also suggested by previous study showing that HEF1, a member

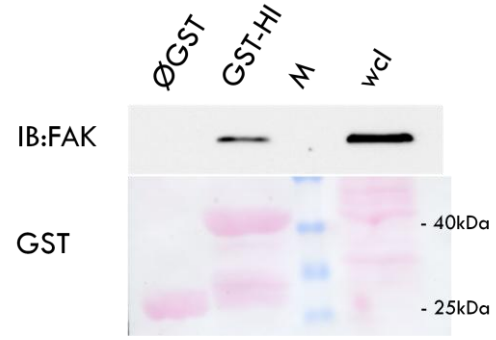
of Cas-family proteins, interacts with itself and that extreme C-terminal part of the protein is responsible for the homodimerization (Law et al., 1996; Law et al., 1999). With the use of pull-down experiment and subsequent immunoblot we showed a very weak interaction of CAS CCH domain with CAS (Figure 5.5A).

5.2.1.3 CAS CCH domain interaction with FAK and vinculin

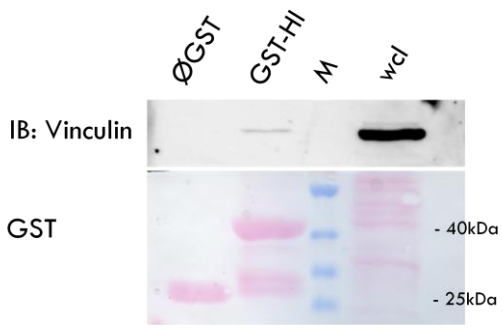
Garron et al., were studying the C-termini of CAS family proteins by three different approaches: biochemical, biophysical and computational. They proved that this domain adopts a four-helix bundle structure reminiscent of those formed by the FAT domain of FAK, by residues 507-631 of α-catenin and by talin domains containing vinculin binding sequence (VBS) 2 and 3 (Garron et al., 2009). Since CAS homodimerize through CCH domain and resembles the FAT domain of FAK and vinculin binding site of talin it is possible that the CCH domain will bind FAK and/or vinculin. These two proteins are stable components of focal adhesions and thus may be responsible for CAS CCH targeting to FA. We therefore did a pull-down experiment with both FAK and vinculin. We showed that CAS CCH domain associates either FAK (Figure 5.5B) but also vinculin (Figure 5.5C).



A)



B)



C)

Figure 5.5 CAS associates with FAK, vinculin and homodimerize with CAS through C-terminal Cas homology domain. Isolated GST-Hl domains were incubated with Hela cell lysates as described in chapter 4.4.2. Interacting proteins were pulled down from the protein lysate, eluted by Laemmli sample buffer and DTT and separated by SDS-PAGE electrophoresis. Proteins were transferred to nitrocelulose membrane that was immunodetected with the use of p130Cas (P130Cas (C-20), sc-860), FAK (FAK (C-20), sc-558), vinculin (Vinculin (N-19), sc-7649) antibodies. As we can see, all three proteins p130Cas (A), FAK (B) and vinculin (C) associate with long variant of CAS CCH domain (top membrane, second lane from left). „Empty" GST was used as a negative control (left lane) and whole cell lysate as a positive control (right lane). GST (~26kDa) and GST-Hl (~46kDa) domains was non-specifically visualized by ponceau stain (bottom membrane). SM0671 protein marker was used (chapter 3.6).

We further analyze the role of CAS, FAK and Vinculin in targeting the C-terminal domain of CAS to FA sites by performing immunofluorescence experiments. However, first we were curious, whether the two variants of CCH domain, short (GFP-Hs) and long (GFP-Hl) differ in localization into FA sites.

MEF cells were transiently transfected with either GFP-Hs or GFP-Hl plasmids and grown on fibronectin coverslips to low density (20–30% confluence). Fixed cells were immunostained for paxillin that served as marker of focal adhesions. Co-localization of the two variants GFP-Hl and GFP-Hs with paxillin was examined with the use of fluorescent confocal microscopy (Leica TCS SP2) and quantified with external Leica software (Leica LCS Lite). To quantify the FA localization of the variants, GFP fluorescence values were determined from measurement of at least fifty FAs. Focal adhesions of robust paxillin staining were chosen for analysis. We showed that long variant of CCH domain GFP-Hl was localized in 80% of focal adhesions while localization of short variant GFP-Hs was impaired to app. 31% (Figure 5.6, Figure 5.7B). This result suggests that the 46 aa long region prior to the helical region is important for localization of CAS CCH domain to FA. Thus long variant of CCH domain was used to test the role p130Cas, FAK and vinculin in targeting this domain into FA.

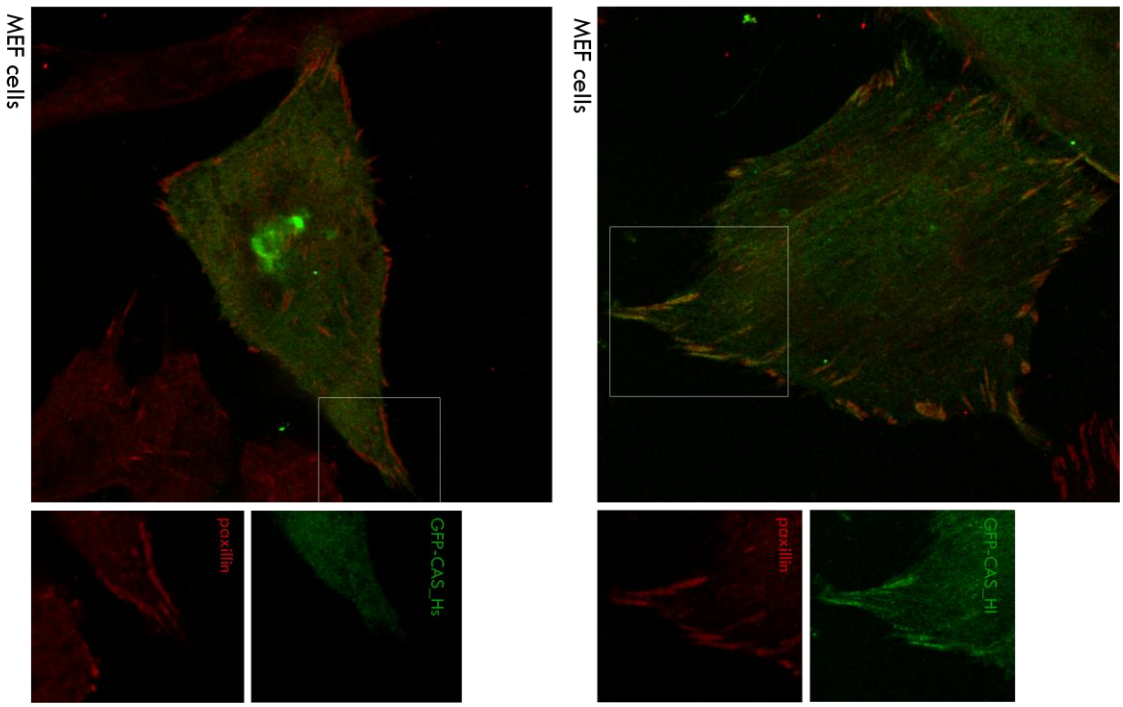
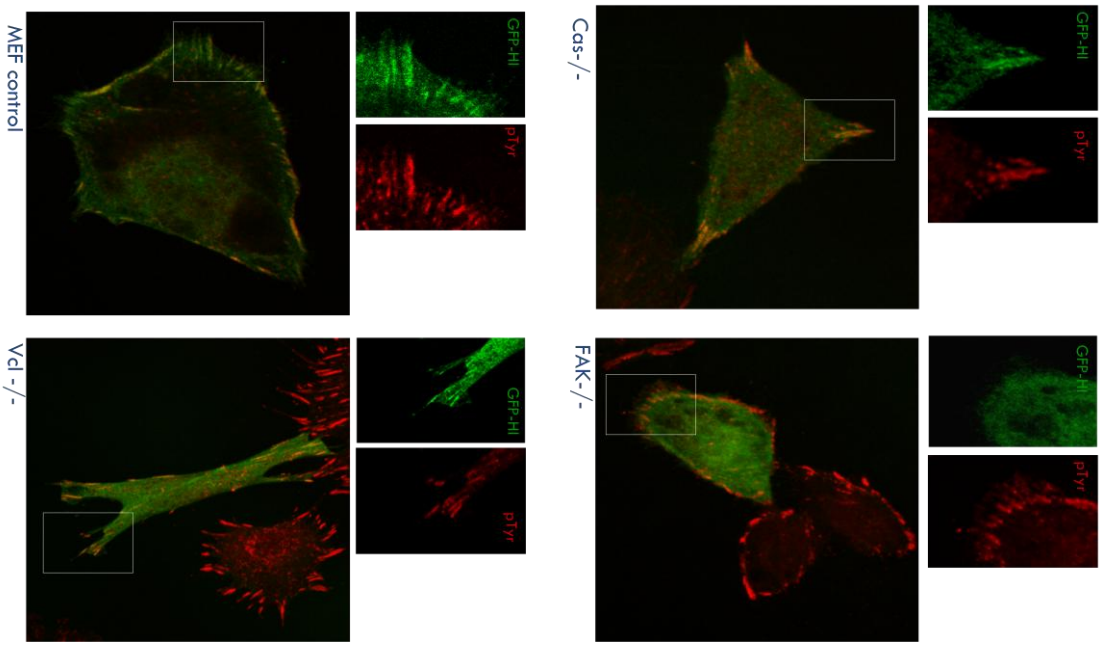


Figure 5.6. A 46 amino-acid-region prior to pure helical part of CAS CCH domain is essential for localizing the CAS CCH into focal adhesions. MEFs were transiently transfected with either GFP-Hs (left) or GFP-Hl (right), grown on fibronectin to low confluence and fixed. GFP-CCH domains are green in cells. Paxillin (red) immunostaining served to mark the FA (Anti-Paxillin 610051). While GFP-Hl was localized either in cytoplasm but mainly at focal adhesion sites, GFP-Hs was localized preferably in cytoplasm. Thus the 46 aa long region that makes difference between the two forms is important for proper localization of CAS CCH into FA sites. Quantification of this result is shown in graph of next figure (5.7B).

The best option how to analyze the effect of CAS, FAK and vinculin on CAS CCH domain localization was to use cell lines derived from mouse embryonal fibroblast but deleted in p130Cas (Cas-/-), FAK (FAK-/-) or Vinculin (Vcl-/-) genes. GFP-Hl plasmid was transfected either to Cas-/-, FAK-/- or Vcl-/- cells and localization of CCH domain in FA was determined as described above. In this case, also phosphorylated tyrosine (pTyr) served as a marker of FA (two different experiments). In comparison to MEF cells that served as a positive control and where the localization of GFP-Hl was app. 80%, the fluorescence of GFP-Hl signal in focal adhesions was decreased in FAK-/- cells (from 80% to 25%) but also in Vcl-/- cells (from 80% to 44%). Expression of GFP-Hl in Cas-/- cells had almost no effect on localization of CAS CCH domain to FA (from 80% to 77%).

A)



B)

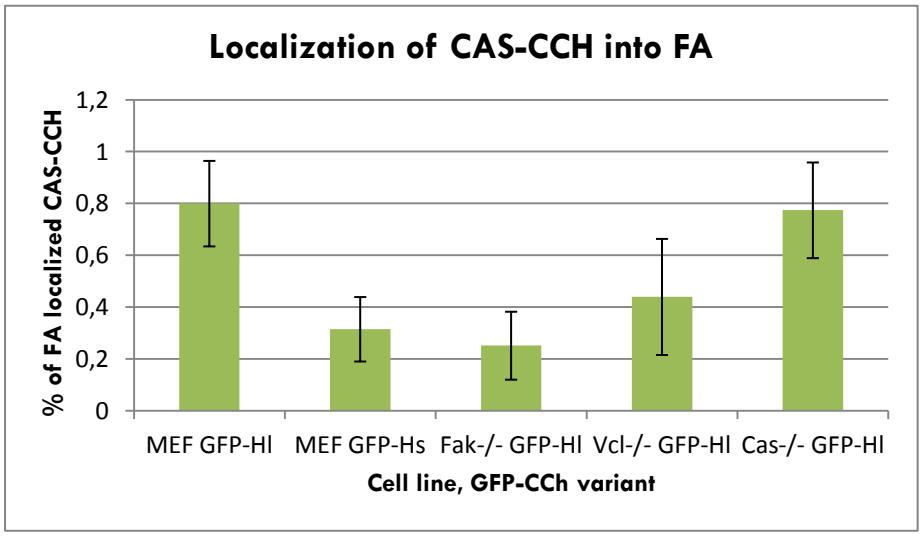


Figure 5.7 FAK and vinculin most probably mediate the localization of CAS CCH long form into FA. (A) The localization of CAS CCH long variant (GFP-Hl) was analysed in four different cell lines: MEF and derived Cas-/-, Vcl-/- and FAK-/- (decsribed always left from each panel). Mouse embryonal fibroblasts (MEF) were used as a positive control of GFP-Hl localization into FA (up left panel). GFP-Hl is shown as green. Cells were immunostained with phosphorylated tyrosine (pTyr) antibody (Anti-Phosphotyrosine clone 4G10®) that served as marker of focal adhesions (red). Original images were merged (the large ones). Brightness is modified by auto-brightness (Microsoft Office Picture Manager, 2007) in detailed images. (B) Localization of CAS CCH domain in FA was quantified as following: At least 50 focal adhesions (marked by paxillin or pTyr, respectivelly) were selected per each variant and cell line respectively and served as a region of interest (ROI). For each FA the mask (ROI) was copied into cell cytoplasm. Level of fluorescence for green and red channel was measured at two different sites of a cell and GFP-CCH localization was counted relatively to Paxillin (GFP-Hs versus GFP-Hl) or pTyr respectively (two different experiments). The graph shows the average level of CCH localization in FA with standard deviation bars.

These results are consistent with previous pull-down assays showing the best association between GST-Hl domain and FAK, weak association of GST-Hl/vinculin and almost no association between GST-Hl and p130Cas (Figure 5.5). Even though interaction between the long variant of CAS-CCH domain and FAK or vinculin was not identified by mass spectrometry results of two different experiments strongly indicate that the two focal adhesion proteins-FAK and vinculin might associate with and thus localize the CAS C-terminal region to focal adhesion.

5.3 CAS CCH domain enrichment in podosome type of adhesions

As described before, the key event by which CAS mediates the downstream signalling is the tyrosine phosphorylation of its substrate domain (SD). In non-transformed MEFs, CAS SD tyrosine phosphorylation is detected predominantly at sites of integrin adhesion including both nascent focal complexes formed at the edges of extending lamellipodia as well as mature focal adhesions underlying the cell body (Fonseca et al., 2004). CAS re-expression in Cas-/- mouse embryonic fibroblasts transformed by oncogenic Src resulted in increased invasiveness of these cells that was caused by activation of matrix-metaloproteinase-2 (MMP-2) and formation of large podosomal structures containing tyrosine phosphorylated CAS (Brábek et al., 2004). SH3 domain of CAS was shown to be required for all processes associated with invasive phenotype of Src-transformed cells but not for formation of podosomal structures (Brábek et al., 2005). Later study investigating the biological significance of tyrosine phosphorylation within CAS SH3 domain showed that phosphomimiking mutation of the SH3 domain Y12E decreased the localization of CAS into focal adhesions but not into podosome-type adhesions (Janostiak et al., 2011).

While studying localization of CAS CCH domain in focal adhesion we observed that the CCH domain localizes also to great number of podosome-type of adhesions. Thus we wanted to estimate what is the localization of CAS CCH domain in podosomes. To study this we used Src-transformed rat sarcoma RsK4 cells that form focal but also significant podosome-type adhesions when grown on fibronectin coated coverslips (Tolde et al., 2010). The podosomes are formed on ventral side of cells and are constituted by F-actin and phosphorylated cortactin core that is surrounded by ring of adhesive molecules (phosphorylated tyrosine or paxillin) (Tolde et al., 2010). RsK4

cells were transiently transfected with long variant of CCH domain and stained for phosphorylated cortactin that served as a marker of podosomes and for paxillin (marker of either the focal adhesions or podosomes). We recorded at least two fold increase of CAS-Hl localization in podosomes when compared to localization in FA (Figure 5.8 A, B).

A) B)

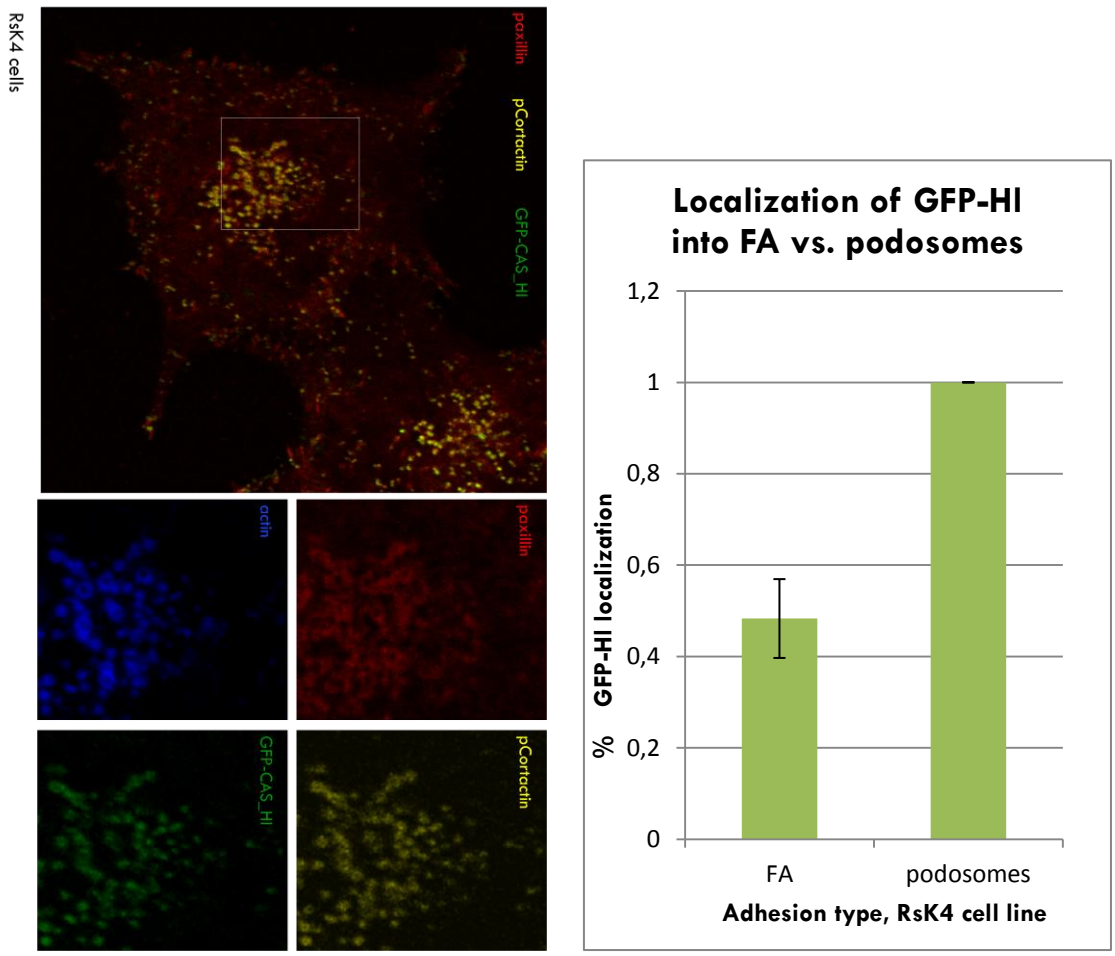


Figure 5.8 CAS CCH domain is enriched in podosome type adhesions when compared to FA localization. (A) RsK4 cells were trasnsiently transfected with GFP-Hl as described above. Fixed cells were stained with paxillin (Anti-Paxillin610051) and phosphorylated cortactin (Anti-Cortactin [pY421]) thus we could mark both adhesive structures. Podosomes were identified as rounded structures at ventral side of cells with core pCortactin surrounded by paxillin boundaries (detailed image). Paxillin elongated structures at the cell periphery were bounded as focal adhesions. It is clearly seen that CAS CCH domain (long variant) localize markedly into podosomes. Actin was stained with phalloidin (DY-405-phalloidin (Dyomics)). (B) The GFP-Hl fluorescence was assessed by fluorescence confocal microscopy and quantified so that the level of GFP protein fluorescence in focal adhesions was counted relatively to the level of fluorescence in podosomes (set as 1, 100%). The graph shows average level of GFP fluorescence in FA with standard deviation bar.

5.4 CAS SH3 domain interaction with vinculin

CAS SH3 domain was identified as the N-terminal region involved in localization (Donato et al., 2010; Harte et al., 2000; Nakamoto et al., 1997) and thus responsible for mechanosensory role of CAS at force sensing focal adhesion sites (Sawada et al., 2006). Several proteins interact with CAS SH3 domains (reviewed in (Defilippi et al., 2006)) of which FAK was described as the main protein mediating the CAS SH3 anchorage. Expression of deletion mutants in FAK-negative cells however indicated that not only FAK but some other proteins may target the SH3 domain into focal adhesions (Donato et al., 2010; Nakamoto et al., 1997). With proteomic approach we were able to identify several new interacting partners of CAS SH3 domain (Table 5.1). Vinculin, as a protein that connects integrins with cytoskeleton was the most interesting result for us. Thus, we decided to study the p130Cas/vinculin interaction in more detail.

To prove the specificity of interaction between CAS SH3 domain and vinculin we performed immunoprecipitation experiments with full length CAS protein. Endogenous p130Cas was immunoprecipitated from Hela cell lysates as described in methods and precipitated CAS and co-precipitated vinculin was detected by immunoblot (Figure 5.9A). The very weak signal of co-precipitated vinculin suggests that the interaction between full-length proteins is weak. This notion is consistent with additional result showing a strong detection of co-precipitated FAK when compared to vinculin (Supporting data S5.6).

To avoid the possibility that vinculin is bound to CAS through FAK, we performed pull-down assay in the absence of FAK. Hela cell lysate was prepared as described before and supernatant was equally separated into two microfuge tubes of ~ 1mg protein amount. The lysates were incubated with or without FAK primary antibody with subsequent protein-A sepharose incubation. The FAK depleted lysate was prepared by centrifuging the immunoprecipitated FAK bound to sepharose beads (Figure 5.9B, top panel, -FAK). Purified GST fused SH3-WT domain was incubated with either FAK positive (+FAK) or FAK negative (-FAK) lysate. We observed that wild type SH3 domain interact with vinculin in the same manner even in absence of FAK (Figure 5.9, middle panel). This result shows that the CAS/Vcl interaction is not mediated by FAK and is most probably direct.

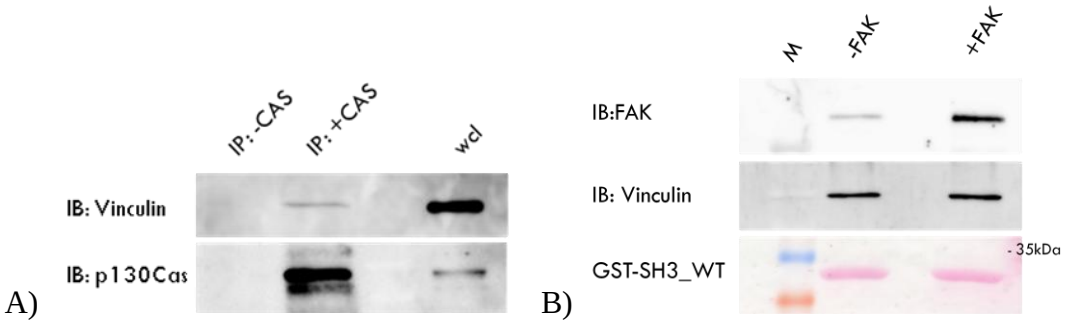


Figure 5.9 CAS SH3 domain associates with vinculin and the interaction is not mediated by FAK. (A) Specificity of CAS-SH3/Vcl interaction was verified by IP of full length CAS**.** Hela cell extracts were immunoprecipitated with p130Cas antibody (P130Cas (C-20), sc-860) using protein A sepharose (GE Healthcare), followed by Western blot analysis using anti-CAS (P130Cas (C-20), sc-860) and anti-vinculin (Vinculin (N-19), sc-7649) antibody. Sample with no addition of primary antibody when precipitated was used as a negative control. By contrast, whole cell Hela lysate was used as a positive control. Immunoblotting shows high efficiency of CAS precipitation (second line, bottom panel) but weak co-precipitation of vinculin (top panel) when compared to positive control (wcl). (B) Pull-down assay was performed using wild type form of GST fused SH3 domain and Hela cell lysate depleted in FAK (left lane -FAK) or not (right lane +FAK). FAK depletion of lysate was verified by immunoblotting (top panel). Middle panel shows that the interaction between CAS SH3 domain and vinculin is not changed even when FAK is decreased in lysate

Predicted binding motif within vinculin is the proline rich "neck" region between vinculin head (V_h) and tail part (V_t). The region (residues 837-878, mouse) contains the PxKP motif that is generally essential for CAS SH3 domain binding (Wisniewska, 2005). GFP-fused constructs of vinculin were created by Dr. Rösel. The wild type form was composed of full length vinculin with N-terminal GFP tag (Vcl-WT), while mutant variant contained a PNSS substitution instead PPKP in the protein "neck" region (Vcl-PNSS). Vcl-/- MEFs were transiently transfected with either GFP-Vcl-WT or GFP-Vcl-PNSS, grown to confluence and lysed. Vinculin was immunoprecipitated from lysates through GFP tag. Immunoprecipitated vinculin and co-precipitated CAS was visualized by chemiluminescence. The PNSS mutation aborted the interaction with CAS so we showed that vinculin "neck" pro-rich region is important for the interaction (Figure 5.10).

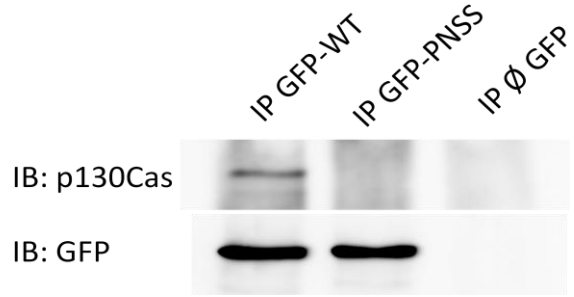


Figure 5.10 The "neck" region of vinculin containing PxKP motif is important for the interaction with CAS. Vcl-/- cells were transfected with either GFP fused wild type (WT) vinculin or a the mutant variant of vinculin which contained a PNSS substitution instead of PxKP in the "neck" region of the protein. Cells were lysed and the lysate was used for an IP. The immunoblot shows that WT variant of vinculin interacts with CAS while the vcl_PNSS mutant does not. "Empty" GFP was used as a negative control.

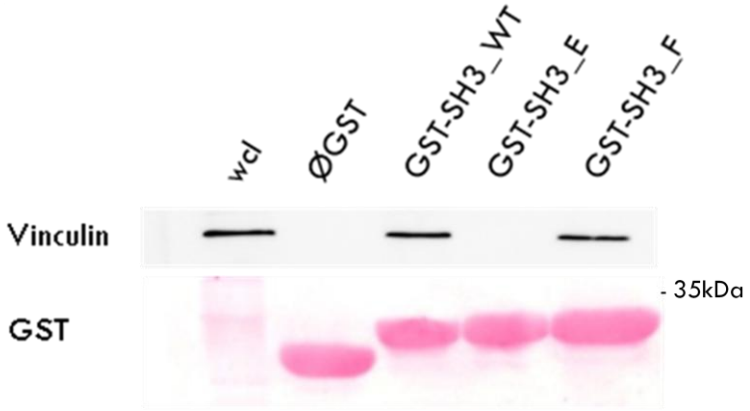
The discovery of new interacting partner of CAS SH3 domain, vinculin led to the collaboration with biophysical laboratory of professor Fabry in Erlangen where the mechanosensory role of the association was further studied.

5.4.1 A phosphomimiking mutation Y12E within SH3 domain of CAS descrease its interaction with vinculin

Recently, a novel phosphorylation site within CAS SH3 domain hydrophobic ligand binding site Y12 was identified in Src-transformed MEFs (Luo et al., 2008). To study the biological significance of the tyrosine phosphorylation, phosphomimiking Y12E and non-phosphorylatable Y12F mutants were created in our laboratory. Using of these two mutants in experiments showed that phosphomimiking mutation resulted in decreased interaction of SH3 domain with FAK and PTP-PEST, reduced tyrosine phosphorylation of FAK and exclusion of CAS from focal adhesion sites. Expression of Y12F in Cas-/- MEFs or Src-transformed Cas-/- cells caused hyperphosphorylation of CAS substrate domain, slower FA turnover and decreased cell migration and invasion respectively (Janostiak, 2011). These results have led us to analyze the binding properties of CAS SH3 domain towards vinculin. We performed pull-down experiments with wild type and mutant forms of GST fused SH3 domains and showed that non-phosphorylatable Y12F decreased the association with vinculin to 72% when compared to wild type (set as 1, 100%). We observed much more prominent decrease (up to 100 fold) when using

phosphomimiking mutant with almost no detectable vinculin association on nitrocellulose membrane (Figure 5.11A, B).

A)



B)

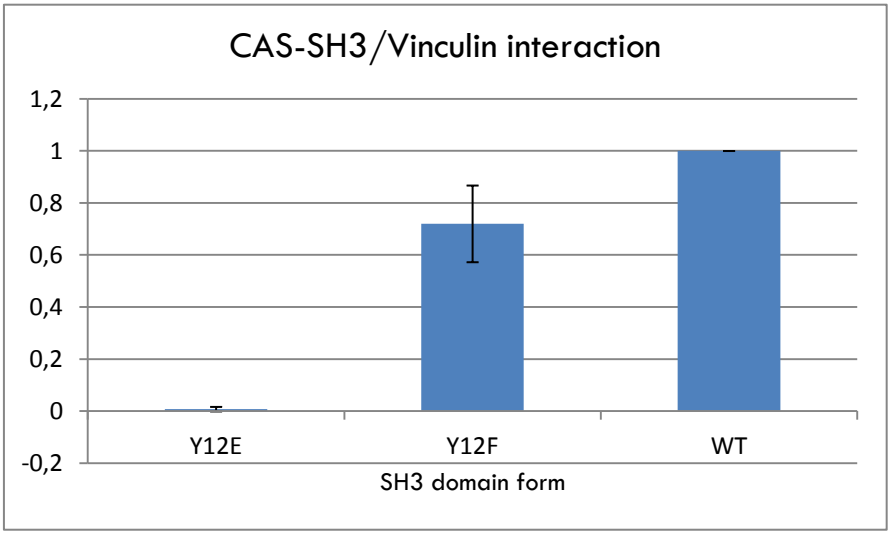


Figure 5.11 Y12E mutation within ligand binding region of CAS SH3 domain disrupts the interaction with vinculin. (A) Pull down experiments were performed with three variants of CAS SH3 domain: WT (wild type), Y12E (phosphomimiking mutant) and Y12F (non-phosphorylatable mutant) that was followed by immunoblotting. Vinculin was detected by vinculin (Vinculin (N-19), sc-7649) antibody (top panel). GST domains were non-specifically stained by ponceau (bottom panel). (B) Signal of vinculin or amount of GST domain from ponceau staining was quantified with the use of ImageJ. The association between SH3 domain and vinculin was counted relatively to amounts of SH3 domains. The interaction between SH3_WT and vinculin was set as 1 (100%). Data of three independent experiments were analyzed and graph shows the average results with standard deviation bars.

These results but also the previous studies of CAS Y12 phosphorylation (Janostiak et al., 2011; Luo et al., 2008) highlight the importance of tyrosine phosphorylation within ligand binding surface of SH3 in regulation of downstream signalling from CAS

protein. Therefore we decided to find out whether regulation through tyrosine phosphorylation is a general phenomenon among SH3 domains. We therefore employed computational tools.

5.5 Survey of SH3 domain phosphorylation

In order to find the abundance of SH3 domain phosphorylation, we queried the publicly available and very comprehensive PhoshoSite Plus (PSP) database through its web interface http://www.phosphosite.org/. We searched for all phosphorylation sites in SH3 domains with the use of Site search option. To the date of the search (October, 2011), Total of 188 phosphorylation sites in 127 SH3 domains originating in human, mouse, rat and chicken proteins were described in database (UniProt codes of proteins with SH3 domain phosphorylation are listed in Table 5.2). A 106 of these were tyrosine phosphorylations (Supporting data S5.7). Since our previous results suggested that tyrosine phosphorylation affects the binding properties of CAS SH3 domain we were focusing only on the tyrosine phosphosites in the further analysis.

Q96B97	Q9BY11	P15498	Q923H2	P59808	Q62696
Q14247	Q9UKS6	P52735	Q08509	NP_001075883	NP_001101686
P46108	P19174	Q9UKW4	P14234	Q62419	Q5I2Z0
P46109	P16885	O60504	P39688	Q8R3V5	Q63622
Q12959	P55345	P07947	Q60631	XP_001474041	P31016
Q12929	Q15700	Q8CBW3	Q8BKX1	Q4ACU6	Q8VI91
P09769	Q9NSI8	P62484	Q03526	Q8K352	XP_001069000
Q96RU3	O94885	Q8BYZ1	Q8VDG6	Q91ZR2	Q9JLU4
P42685	Q5HYK7	P00520	Q9Z279	Q62417-5	Q04929
P06241	Q99961	Q7TQF7	O55033	P16546	
O75791	A1X283	XP_901719	Q9DC07	P05480	
P62993	Q7Z6J0	Q8VHL8	Q6WKZ7	NP_001020566	
P08631	O15117	P35991	Q61140	P27870	
Q9UHR4	O75995	O70589	Q61644	Q9R1Z8	
Q08881	Q13813	Q6P9K8	Q99JB8	Q04736	
Q9NZM3	P12931	Q8R550	Q8CIH5	O08838	
P06239	P42680	Q64010	Q91XM9	P54283	
Q5TCX8	Q5TCZ1	P47941	Q62108	Q8VHK2	
NP_001073996	Q13470-2	Q811D0	Q80U22	Q63768	
O43639	P42681	P70175	P57725	Q5U2U2	

Table 5.2 UNIPROT codes of proteins with SH3 domain phosphorylation. List of proteins that contain a phosphorylated SH3 domain (the outcome of PSP database to October, 2011).

5.5.1 **Identification of the tyrosine phosphorylation sites within SH3 domains**

For the list of tyrosine phosphorylated SH3 domains generated by PhosphoSite Plus we further verified whether the phosphorylation sites really occurred within SH3 domain. All the sequences of SH3 domains were obtained and their length was defined using the domain definition SMART tool (Letunic et al., 2009; Schultz et al., 1998). To study the abundance of specific tyrosine phosphorylation in SH3 domain we generated a multiple sequence alignment (MSA) from verified data (Figure 5.12). To avoid redundancy, first we excluded all the orthologue and paralogue (isoform) sequences with identical phosphorylation footprint. Fifty-two SMART based SH3 domain sequences were aligned using ClustalW (Thompson et al., 1994). A total of 36 protein domains were phosphorylated at one tyrosine site, 15 of them at two tyrosine sites and one (PLCγ2) at three sites. When looking at the phosphotyrosine conservation we found twenty phosphorylated tyrosines in two orthologue domains (Supporting data S5.8) and eight phopho-tyrosines in three orthologue domains (Supporting data S5.9).

To characterize the abundance of tyrosine phosphorylation at each tyrosine position in SH3 domain, we numbered each amino acid in sequence according to the alignment in Figure 5.12, we showed that most tyrosine phosphorylations occurred at positions seven (Y7) and sixty-six (Y66) (Table 5.3). These positions correspond to Y90 and Y131 in chicken Src SH3 domain and are localized in the first and the third hydrophobic pocket that are involved in ligand binding (Erpel et al., 1995; Xu et al., 1997).

5.5.2 **Analysis of phospho-enriched positions**

To analyze the consensus sequence surrounding of the two phospho-enriched positions we used web-based application WebLogo (Crooks et al., 2004). We selected sequences with phosphorylation with the two most abundant positions (position 7 and position 66) and created a nine amino-acid-long sequence logos (Figure 5.13) that reflected sequence conservation at each of these positions. There was no stronger amino acid conservation around the Y66 (with the exception of Proline on the position +2). However, the sequence surrounding around the Y7 was very well conserved with motifs AL**Y**DY or AL**Y**DF.

Figure 5.12 Multiple sequence alignment of tyrosine-phosphorylated SH3 domains. This alignment was performed using ClustalW and includes sequences of all SH3 domains phosphorylated at tyrosine sites. Phosphorylation sites within SH3 domains were searched by database Phosphositeplus. All the sequences were obtained and their lenghts were defined using SMART server. Names of proteins according to UniProt database are situated on the left including the domain range if there are more than one SH3 domains within a protein. Human sequences are shown except from those depicted with _m that come from mouse. Alignment is numbered at the top. Phosphorylated tyrosines are highlithed in red.

Phospho-Y position within SH3	2	3	**7**	9	11	17	30	31	35	42	49	50	51	54	55	56	59	64	**66**	71
Number of phoshorylations detected per site	2	2	**24**	6	1	6	2	1	2	1	1	1	1	1	3	1	1	1	**11**	1

Table 5.3 Position-based phosphotyrosine abundance within SH3 domain. Phosphotyrosine position within SH3 refers to the position in alignment in Figure 5.12. Out of fifty-two SH3 domain seguences, the two most abundant phosphorylated sites were Y7 that was phosphorylated 24 times and Y66 that was phosphorylated 11 times.

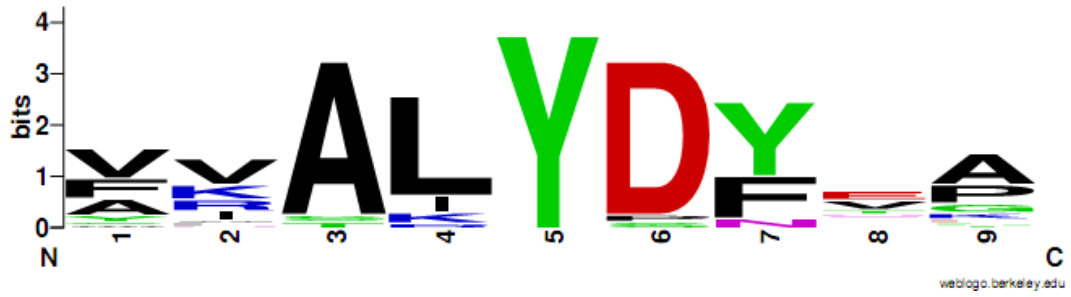
Next, we wanted to find out how many of the human SH3 domains contain this AL**Y**DY/F motif. Based on SMART database, out of 304 human SH3 domains, the AL**Y**DY motif around Y7 appeared in 21 domains and ALYDF motif in 15 domains. Of those 36 sequences (Supporting data S5.10), 12 domains are known to be phosphorylated at Y7 according to Phosphosite Plus. This means that approximately 12% of the all SH3 domains in SMART database possess sequence motif around tyrosine 7 that can be potentially phosphorylated. We gained similar data from Pfam database (Finn et al., 2010): out of 750 human SH3 domains there are 113 sequences (~15%) with either AL**Y**DY (64) or AL**Y**DF (49) motif (Supporting data S5.11 and Supporting data S5.12).

5.5.3 **Tyrosine phosphorylation is enriched in other docking domains**

According to statistics 13324 (21.4%) pY, 11618 (18.6%) pT (phosphorylated threonine) and 37410 (60%) pS (phosphorylated serine) human sites have been made public in PhosphoSite Plus to October 2011. This means that only fifth of all phosphorylation sites within proteins are tyrosine-phosphorylations. However, when considering our search for tyrosine phosphorylation within SH3 domain, we could see that the ratio of this type of phosphorylation is much higher (56.4%, first search). Thus we wanted to find out whether prevalence of tyrosine phosphorylations in docking domains is a general phenomenon. We chose eight docking domains SH3, SH2, PH, PDZ, WW, PTB, EH, PX and using Phosphosite Plus database we searched for phosphorylation sites within these domains separately. To avoid redundancy we used only human proteins. For each domain we counted the ration of tyrosine-phosphorylated

sites to all of them (pY + pT + pS). The prevalence of phosphorylated tyrosines in adaptor domains compared to the whole human proteome was normalized to number of tyrosines in the domain or human proteome eventually (Table 5.4). Except from PTB domain that contained no tyrosine phosphorylation, in all of the docking domains was the tyrosine phosphorylation enriched when compared to the whole proteome tyrosine phosphorylation. The difference could be mostly obvious in case of Ef Hand (EH) domain (6.1 fold increase) but also in case of PDZ (3.3), SH3 (2.2) and PH (2.1) domain. The results gained of Ef hand domain are questionable since there have been detected only two phosphorylation sites within this domain and these were tyrosine phosphorylations. The same issue applies to the PTB domain with the only one serine phosphorylation.

A) 24



B) 11

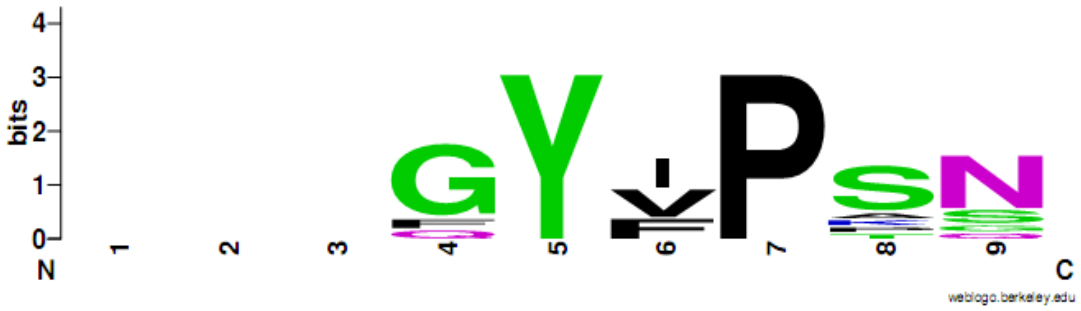


Figure 5.13 Weblogo of aligned segments of SH3 domains phosphorylated at Y7 and Y66 sites. Based on previous Multiple sequence alignment. Sequences with the most abundant pY sites either (A) pY-7, (B) pY-66 were selected from previous MSA. Sequence logos were created by 9aa long alignments. The numbers of sequences for each WebLogo are indicated. To generate the sequence logos here we used WebLogo (available at http://weblogo.berkeley.edu/logo.cgi).

Domain	Number of pY to all phosphorylations	Ratio of pY to all phosphorylations	Average Number of tyrosines (%)	Prevalence of pY
Human proteom	13324/62352	21,4%	2,6	**1,0**
SH3	114/163	69,9%	3,8	**2,2**
SH2	136/193	70,5%	5,3	**1,6**
PH	218/337	64,7%	3,7	**2,1**
PDZ	40/123	32,5%	1,2	**3,3**
WW	22/32	68,8%	7,2	**1,2**
PTB	0/3	0,0%	1,5	**0**
EH	2/2	100,0%	2	**6,1**
PX	45/86	52,3%	3,7	**1,7**

Table 5.4 Prevalence of phosphor-tyrosine to all phosphorylations within selected adaptor domains. Phosphorylated domains were searched separately by PhosphoSite Plus. Only human phospho-sites were used in the analysis. The ratio of phosphotyrosine sites to all (pY, pS and pT) sites were counted. The phosphor tyrosine enrichment in adaptor domains was determined with regards to the ratio of tyrosines in the domains (human proteome respectively).

6 Discussion

In this study we were focusing on two different signalling domains using both experimental and computational methods.

P130Cas protein, an adaptor protein of integrin signalling is in the center of interest of our group for several years. This protein has been linked to processes such as migration, adhesion, apoptosis, cell cycle, differentiation and survival in normal and pathological cells (reviewed in (Defilippi et al., 2006; Tikhmyanova et al., 2010)). In all of these processes p130Cas function as a multi-domain signalling module integrating diverse stimuli that are essential for cell communication in higher organisms. Protein-protein interacting domains of p130Cas are also responsible for mechanosensory role of this protein at focal adhesion sites of cells (Donato et al., 2010; Fonseca et al., 2004; Harte et al., 2000; Nakamoto et al., 1997; Sawada et al., 2006). More specifically, the anchorage of p130Cas to cell membrane through N-terminal SH3 domain and extreme C-terminal CCH domain is the starting event in p130Cas mechanosensory role. The main aim of this study was to search for CAS partners that could mediate the localization of the protein to focal adhesions. We performed standard proteomic experiments and used mass spectrometry analysis to identify new associating proteins of CAS. We further focused on proteins that were interesting for us with respect to localization in cells.

6.1 A forty-six amino-acid-region prior to four-helix bundle is important for the proper function of CCH domain in localizing CAS into FA

The CCH domain has been shown to be a highly hydrophobic four-helical bundle (Garron et al., 2009; Law et al., 1999; Mace et al., 2011) and thus seemed very challenging from biochemical aspect of view. By optimizing the purification conditions we were able to isolate the longer variant of CCH domains (GST-Hl) in relatively big amounts but have continuous difficulties with isolation of the short variant (GST-Hs).

The two variants of CCH domain also differed in localization in cells that was seen of fluorescence experiments. The results showed that the short variant that covers only the four-helix bundle (Helix short, Hs) is insufficient for localization of the domain into FA and that additional sequence prior to the helical region (Helix long, Hl) is necessary for

the FA targeting. Expression of GFP-fused CCH domains in mouse embryonic fibroblasts showed up to threefold decrease in GFP-Hs when compared to long variant (GFP-Hl). There are two possibilities how to explain this result. First, the 46-amino acid long region is important to organize structurally the helical domain and only this compact domain can properly function in cells. The other explanation is that the 46-aa long region is the sequence that targets the domains into FA.

Donato et al., in her study used deletion mutants to characterize domains responsible for targeting CAS into focal sites. She used deltaCCH deletion mutant that lacks the last C-terminal 141 amino acids. This lacking region roughly correspond to our CAS CCH domain – short variant. DeltaSH3 (SH3 domain is the first described FA targeting domain of CAS) mutant decreased the localization of CAS to FA but only the double mutant deltaSH3/deltaCCH resulted in full exclusion of CAS from focal adhesions (Donato et al., 2010). From this we could conclude that the 46 amino-acid-region of CAS-Hl domain is not the localizing domain into FA otherwise the double mutant deltaSH3/deltaCCH would have to localize to FA at least partially as it contains a vast majority of 46-aa region. We could therefore conclude that the 46 amino-acid-region is most probably necessary to organize structure of four-helix bundle that is responsible for localizing CAS to FA.

This notion can be strengthened with studies that were focusing on folding of domains in multidomain proteins (Bhaskara and Srinivasan, 2011; Han et al., 2007). Domains of multidomain proteins considered in isolation are significantly less stable that the single domain homologues. On the contrary full-length multidomain proteins have similar stability of those single domain proteins, suggesting the role of interdomain interactions in stabilizing the multidomain proteins. Most interdomain contacts are made of hydrophobic residues like Leu, Ile, Phe, Ala and Tyr that create energetically favored interactions on the domain:domain interface. The interfacial stabilizing residues are better conserved, have substantial intra-protein contacts, are located more deeply, have less protrusion into the solvent surrounding. The destabilizing effects on multidomain proteins were mostly caused by mutation of such a non-polar residue to a polar or a mutation of residue on ligand-binding surface. The large number of hydrophobic residues at domain:domain interface would be exposed to the solvent in absence of the other domain which is energetically unfavorable (Bhaskara and Srinivasan, 2011; Han et al., 2007; Zhou et al., 2004). We could also speculate that the 46-aa region serves

instead of an interdomain contact and helps the CCH domain to gain a compact four-helical fold when expressed in cells in isolation. The same thing can also happen when the CCH domain interact with another domain *in trans*.

Last year Mace et al., solved the crystal structure of CAS CCH domain in complex with an NSP3 protein domain and provided the first atomic-resolution evidence that the CCH domain of CAS adopt a well-defined FAT domain-type four-helical bundle. They showed an extensive binding interface and a very tight interaction between the two domains. Even single mutations of hydrophobic residues on binding interface markedly reduced association of the two proteins (Mace et al., 2011). It could be that the CCH that is otherwise unstructured but upon interaction with other helical domain become more organized and fall to energetic minimum which could also explain the reason why the interaction of CAS-CCH/NSP3 complex was so tight. Garron et al., studying similar complex HEF1/BCAR3 described a very poor solubility of the domains when expressed and purified separately. By contrast, when the two domains were expressed independently but co-purified by mixing two cell cultures before lysis it was possible to obtain pure and highly soluble complexes. The purification of the domain could not be circumvented by reducing agents or high salt solutions assuming that the low solubility of these domains when expressed individually was not due to an atypical fold but was caused by non-specific associations of ´´sticky´´ surface areas that are buried in the complex after association (Garron et al., 2009). Similarly, neither we could avoid aggregation and improve the purification capacity by higher percentage of salts (200-400mM NaCl) in case of the short variant of CCH domain. On the other hand, the high molarity of salts optimized the purification of the long variant GST-Hl. This result gave a rise to another possible role of the 46-aa long region in which it can block the self-assembly of the hydrophobic helical bundle and prevent domain aggregation in solution. The other possible role stays the same that it actively helps to fold the needed structure so it is possible to purify the domain. Further studies need to be done to solve the problem which of the two possibilities is right.

Structural analysis of Focal adhesion targeting (FAT) domain of FAK revealed two hydrophobic patches on the opposite sides of the helical bundle that are responsible for binding its interacting partner, paxillin. Each patch is formed at the interface between two helixes, where hydrophobic side chains on the surface of helixes extend the hydrophobic core of the bundle (Hayashi et al., 2002). Mutational analysis showed that

either of the hydrophobic patches at the same time is sufficient for paxillin binding (Bertolucci et al., 2005; Hayashi et al., 2002) (Figure 6.1).

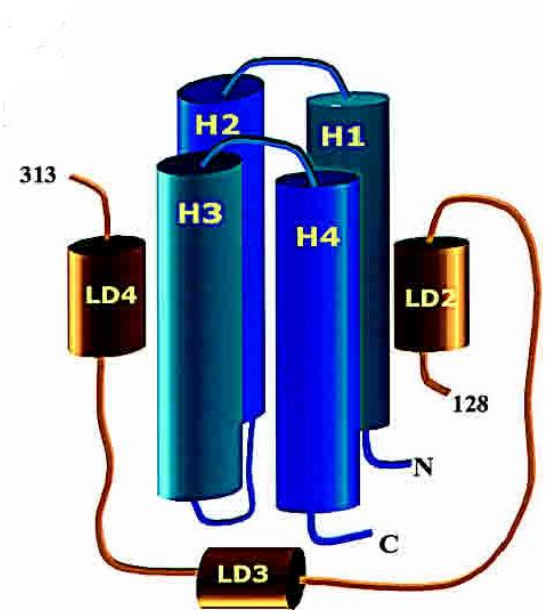


Figure 6.1A model of paxilin helixes binding to FAT domain of FAK, schematic presentation. The paxillin motifs (LD2, LD4) bind to opposite faces of four-helix bundle of FAT. The model serves as a basis for better understanding the idea of CAS CCH domain interaction *in cis* and *in trans* (Bertolucci et al., 2005).

Comparing this with the structure of CAS CCH domain these two hydrophobic patches/grooves are created by helixes α1/α4 and helixes α2/α3. The α2/α3 hydrophobic binding surface is responsible for interaction with GEF-like domains (Garron et al., 2009; Mace et al., 2011) (Figure 6.2) and most probably also for the interaction with new discovered interactors FAK and vinculin. The role of the other side of four-helical bundle remains unclear.

We could speculate that a 46-aa region of CCH-Hl contacts the α1/α4 side of four-helical bundle thus inducing the favorable stable conformation of the CCH domain and the interaction with associating proteins follows. It would be interesting to see whether a mutation on α1/α4 side potentially binding the 46-aa region would result in loss of an association of the CCH domain with its interacting partner that is actually mediated by the opposite face of the domain (α2/α3). For mutational analysis amino acids within α1/α4 hydrophobic groove that are non-polar, well conserved, are located more deeply and has less protrusion into the solvent surrounding should be chosen and substituted

for polar amino acids. All these speculations should be based and combined with bioinformatic analysis or modeling. Also CCH domains interactions *in trans* could be bioinfomatically modeled and verified by mutational analysis. All these experiments would help us to find out the process of CAS CCH domain arrangement and mechanism of the CCH domain in association also with the newly identified interacting partners.

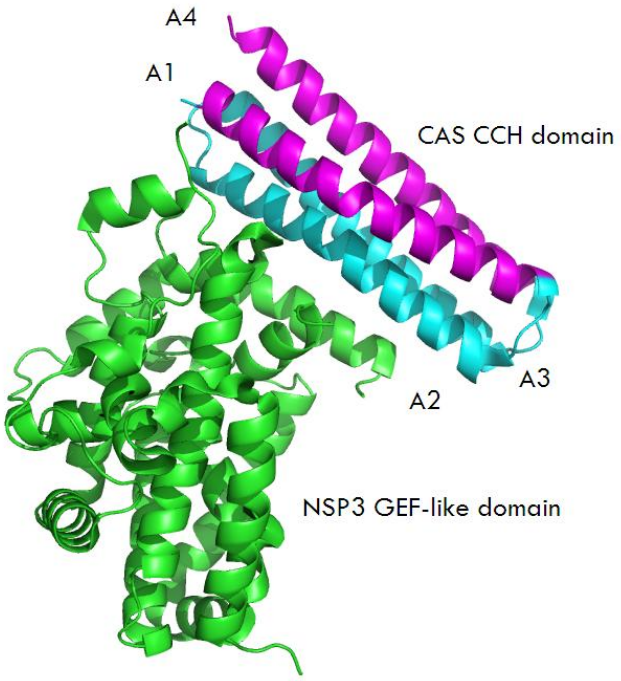


Figure 6.2 The crystal structure of CAS CCH domain in interaction with GEF-like domain of NSP3. NSP3 GEF-like domain is shown in green. The hydrophobic groove between helix α2 and α3 (A2 and A3, shown as cyan) of the CCH domain four-helix bundle is mediating the interaction with NSP3 protein. Picture created by The PyMOL Molecular Graphic System (version 0.99). PDB code of the structure is 3T6G (Mace et al., 2011).

6.2 Anchorage mechanism of p130CAS into focal adhesions through the CCH domain

Difficulties with CAS CCH domain purification led also to very poor outcome from the MS analysis. We identified two proteins that were suggested to interact with CAS CCH domain: BCAR1 and tubulin.

We confirmed a very weak interaction with BCAR1 which is the human homologue of p130Cas (Figure 5.5A). Since HEF1, another member of Cas-family proteins also interacts with itself and the CCH domain is responsible for the homodimerization (Law et al, 1996; Law et al., 1999), it is very possible that CAS will homodimerize through

the CCH domain as well. This offers a way to a possible model of CAS anchoring to FA (Figure 6.3). While the SH3 domain is responsible for physical anchorage of CAS protein to cell membrane the CCH domain creates a supposed “bridge”. This manner of anchoring does not change the basic model in which the end points of CAS SD (SH3 domain and CCH domain) are responsible for the stretch of the SD resulting in increased tyrosine phosphorylation and downstream signalling upon cell stretch (Figure 2.5).

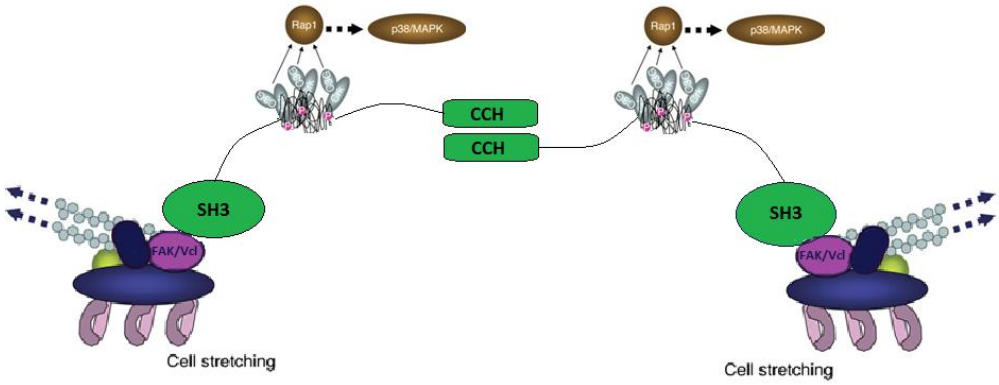


Figure 6.3 A model of CAS anchorage in FA when homodimerizing through the CCH domain. The identification of CAS as interacting partner of CAS CCH domain led to assumption that this domain is responsible for homodimerizeation of CAS. A possible arrangement of CAS anchorage to cell membrane is the following.: CAS is anchored to cell membrane only through the SH3 domain at the N-terminus of the protein. Here, FAK and the newly identified vinculin are mediating the anchoring. C-terminal CCH domain is not directly (by interaction with a FA protein) anchored to cell membrane but homodimerize and creates a supposed “bridge”.

We were not able to confirm the interaction between CAS CCH domain and tubulin. It is very possible that the outcome of MS analysis was the result of tubulin high abundance in cells. By contrast, it would be interesting to see whether proteins that either bind tubulin and are localized in FA would associate with CAS CCH domain For example, Integrin-linked kinase (ILK) is a member of a multiprotein complex at focal adhesions and has been shown to interact with tubulins (Fielding et al., 2008).

Based on previous studies describing the structure of CAS CCH domain but with no MS verification we showed the association of this domain with two proteins of focal adhesions: FAK and vinculin. Expression of GFP tagged CCH-Hl domain in FAK null

and vinculin null cells showed the importance of these two proteins for localizing the CAS CCH domain into focal adhesions. Thus, we were the first to identify proteins that interact with CAS CCH domain and are known as typical markers of focal adhesions at the same time.

6.3 CAS CCH domain localizes more likely into podosome-type adhesions

While studying the CAS CCH domain anchorage to cell membrane at FA sites we reported that this domain also very likely localizes to adhesive structures different from FA – podosomes. RsK4 cells are Src-transformed rat sarcoma cells that form great number of both podosomes and focal adhesions. Expression of GFP-CCH-Hl domain in RsK4 cells revealed at least two-fold increase in CCH domain localization into podosomes when compared to focal adhesion localization suggesting a novel role of CCH domain in other than focal-type of adhesions.

It has been previously observed that CAS over-expression in Src-transformed Cas -/- cells caused organization of podosomes into large aggregates that appear as internal rings, partial rings and peripheral belts. CAS substrate domain was shown to be physically associated with the large podosome rings and belts with the use of antibody that specifically recognizes phosphorylated YXXP tyrosines in CASSD (Fonseca et al., 2004). Mutational analysis showed that CAS substrate domain but not the SH3 domain are required to promote formation of large podosome structures (Brábek et al., 2005). Neither the Y12F nor Y12E within CAS SH3 domain had influence on the CAS localization into podosome-type adhesion (Janostiak et al., 2011) further indicating a possible role of CAS CCH domain in podosomes. An experiment that could partially confirm this notion is the re-expression of CAS CCH domain in Src-transformed Cas-/- cells and examination of the large podosome rings and belts formation when compared to non-transfected cells or cells transfected with type CAS.

6.4 Anchorage mechanism of p130Cas into focal adhesions through SH3 domain

SH3 domain is one of the best described protein interaction module with highly organized globular structure when compared to the CCH domain. By mass spectrometry we were able to identify several previously known but also novel interacting partners of p130Cas SH3 domain. The most interesting result was the association with vinculin. We showed that the interaction CAS-SH3/vinculin is not mediated by FAK which was the only known CAS SH3 domain FA-targeting protein for long time (Nakamoto et al., 1997; Polte and Hanks, 1995). We also proved the specificity of the interaction which was actually further studied in detail by mutant variants. Vinculine mutant (Vcl_PNSS) served to prove that the proline-rich neck region of vinculin is important for the interaction. Tyrosine 12 within CAS SH3 domain has been shown to be phosphorylated in Src-transformed cells (Luo et al., 2008). A phosphomimiking mutant Y12E was created in our laboratory to study the effect of this modification on SH3 domain binding properties. The Y12E of CAS SH3 domain resulted in total disruption of the interaction with FAK (Janostiak et al., 2011) and we showed the same result in case of vinculin. However, analyzing the association of full-length CAS with Y12E mutation, the signal of immunoblot decreased only twice (Supporting data S6.1). This suggests that some other part of CAS protein is associating with vinculin. Later we observed that also CAS CCH domain binds vinculin. Complications during the IP experiments and poor immunoblot signals (Figure 5.9A and Supporting data S5.6) when compared to CAS/FAK interaction suggest a low affinity association between CAS and vinculin.

6.5 Tyrosine phosphorylation as a modulator of protein:protein interactions

Adaptor domains like SH2 or SH3 were first identified as folded structures without enzymatic activity within tyrosine kinases. They were proposed to modify kinase activity by substrate recognition that was important for cellular transformation (Mayer et al., 1991; Sadowski et al., 1986). The main role of adaptor domains is mediating protein:protein interactions, organizing cell pathways and networks and thus regulating cellular responses (Pawson, 1988; Pawson and Nash, 2003). Since the beginning, cloning experiments revealed that these domains are usual elements of seemingly

disparate proteins (Stahl et al., 1988; Trahey et al., 1988). This further reinforced the importance of adaptor domains.

It has been 57 years since the breakthrough study on the conversion of phosphorylase b to phosphorylase a, by Fischer and Krebs, that first emphasized the significance of reversible phosphorylation in biology (Fischer and Krebs, 1955). Nowadays, we know that phosphorylation is the key regulatory step in signalling pathways responsible for cellular development and differentiation, cell cycle control, metabolism, and the immune response. Traditionally, tyrosine phosphorylation is thought to facilitate protein:protein interactions through recognition of phosphor-tyrosine by a protein containing SH2 or PTB domain. This leads to propagation of downstream signalling. However, tyrosine phosphorylation can also regulate catalytic activity either through intermolecular interaction between pY moiety and an SH2 domain (Src kinase) or through an allosteric mechanism (PLCgamma) (Hunter, 2009).

6.5.1 **Tyrosine phosphorylation of adaptor SH3**

However, there is a growing body of evidence on SH3 domain tyrosine phosphorylation that either prevents or reduce the affinity of domain:protein interactions. This often further result in more complex change in cell behavior (summarized in Table 6.1) as it was in case of c-Abl SH3 domain phopshorylation in CML cells which caused enhanced cell transformation potential (Chen et al., 2008; Meyn et al., 2006). In down-regulated state the c-Abl SH3 domain of the kinase mediates interaction with SH2-kinase linker that forms a PPII helix thus keeping c-Abl inactive (Nagar et al., 2006; Nagar et al., 2003). SH3-SH2 region of the protein has been reported to be strongly phosphorylated by Src family kinases (Hck, Fyn and Lyn). SH3 domain Y89 was the most prominent phosphorylation site in vitro in CML cells (Meyn et al., 2006). Phosphorylation of the Abl SH3 domain on Tyr89 interfered with binding to regulatory adaptor protein Abi-1 *in trans* but also decreased the association with SH2-kinase linker *in cis* leading to partial c-abl activation and enhanced cell transformation potential (Chen et al., 2008; Meyn et al., 2006).

Protein	non-phospho-rylatable mutation	phospho-mimicking mutation	phospho-tyrosine	corresponding position in the alignment	Effect of mutation/phosphorylation	Ref.
Abi-1			Y398p	Y7	reduces binding to Abl	(Sato et al.,
Abl	Y89F			Y7	decreases Bcr-Abl-mediated transformation of TF-1 myeloid cells to cytokine independence	(Meyn et al., 2006)
			Y89p	Y7	decreases interaction of SH3 domain with binding partners both in *cis* and in *trans*	(Chen et al., 2008)
Btk	Y223F			Y7	blocks Btk autophosphorylation and potentiates the transforming activity of Btk in fibroblasts	(Park et al., 1996)
			Y223p	Y7	disrupts the interaction with WASP	(Morrogh et al., 1999)
Crk			Y251p	Y17	induces Abl kinase transactivation	(Sriram et al.,
Grb2			Y209p	Y71	reduces binding to Sos	(Li et al., 2001)
Itk	Y180F			Y7	plays positive role in Itk signalling	(Wilcox and
p130CAS	Y12F			Y7	decreases invasiveness in Src-transformed cells	(Janostiak et al., 2011)
		Y12E		Y7	decreases interaction of SH3 domain with FAK and PTP-PEST	(Janostiak et al., 2011)
			Y12p	Y7	decreases interaction of SH3 domain with FAK	(Janostiak et al., 2011)
PST-PIP		Y367E		Y7	decreases interaction with WASP	(Wu et al., 1998)
Endophilin		Y315E		Y7	decreases interaction of SH3 domain with Dynamin	(Wu et al., 2005)
ADAP			Y559p	Y66	positively affects interaction with Nck protein	(Sylvester et al., 2010)
CAP	Y623F			Y7	results in partial nuclear localization of CAP protein	(Fernow et al., 2009)
Src	Y90A, Y92A			Y7, Y9	disrupts the interaction with Sam68 and PI3K-p85α	(Erpel et al., 1995)
	Y133F, Y138F			Y66,Y71	inhibit PDGF and EGF mitogenic signalling	(Broome and Hunter, 1996)
Txk			Y91p	Y7	contributes to upregulated IFN-g gene transcription	(Kashiwakura et al., 2002)
Vav1	Y826F			Y55	reduces binding to CSK	(Lazer et al.,

Table 6.1 Summary of effects caused by mutation or phosphorylation at tyrosine sites in SH3 domains.

Growth factor receptor-binding protein-2 (Grb2) is an adaptor protein in Bcr/Abl initiated signalling. Tyr209 within C-terminal SH3 domain of Grb2 was identified as a

tyrosine phosphorylated site and the phosphorylation resulted in reduced binding of Grb2 SH3 domain to Sos proline-rich peptide in vitro (Li et al., 2001).

Rather than direct effect on binding properties of SH3 domain had tyrosine phoshorylation in Txk SH3 domain. Auto-phosphorylation at Y91 contributed to promoted interferon gamma synthesis (Kashiwakura et al., 2002). FAK mediated Src phosphorylation of endophilin A2 at Tyr315 which is situated at protein ligand binding site blocked the endophilin/dynamin interaction that subsequently led to inhibition of MT1-MMP endocytosis and promoted extracellular matrix degradation (Wu et al., 2005).

Furthermore, a shotgun phosphotyrosine proteomics strategy was used to compare phosphotyrosine profiles from Src transformed cells and their non-transformed counterparts. Of particular importance were Y90 site in Src and homologous Y12 in Src-substrate CAS (Luo et al., 2008). This tyrosine is situated on the ligand binding site of SH3 domain and with the use of phosphomimiking and non-phosphorylatable mutants is intensively studied in our laboratory for both Src and CAS SH3 domain. The known effects of either tyrosine phosphorylation or mutations on SH3 tyrosines are summarized in Table 5.1. The experimental records collected on the SH3 domain tyrosine phosphorylation highlights the importance of tyrosine phosphorylation in regulation of SH3 mediated interactions and signalling, however, it also shows a very unorthodox effect of tyrosine phosphorylation.

This novel regulatory effect of tyrosine phosphorylation on an adaptor domain is not restricted to SH3 domain. Src mediated tyrosine phosphorylation of tensin-3 SH2 domain contribute to biological activity of this protein that is important in cell migration, anchorage-independent growth, and tumorgenesis in lung cancer, breast cancer, and melanoma cell lines (Qian et al., 2009).

6.5.2 Tyrosine phosphorylation is enriched within ligand binding surface of SH3 domain

In order to find out how often is tyrosine phosphorylated within SH3 domain we searched manually curated and comprehensive Phosphosite Plus database (Hornbeck et al., 2004) that collects posttranslation modification of proteins. During our

bioinformatic analysis entries in the database were modified several times confirming that this is really a dynamic and continuously verified source. We identified more than a hundred of tyrosine phosphorylated sites within human, mouse, rat and chicken SH3 domains and processed the results so that they were not redundant (multiplied). By creating a sequence alignment of phosphorylated SH3 domain sequences we were able to determine the abundance of homologous sites and showed that two mostly phosphorylated tyrosine sites correspond to position Y7 and Y66 in the alignment. Both of these residues are situated on ligand binding surface of SH3 domain thus emphasizing the possible impact of tyrosine phosphorylation at these sites. More specifically, Y7 is homologous to chicken Src Y90 that is situated in the first binding pocket while Y66 homologous to Y131 is located within the third binding pocket (Erpel et al., 1995; Xu et al., 1997).

The most commonly phosphorylated tyrosine with 24 occurrences among 52 SH3 domain sequence was the tyrosine at position seven. We decided to better characterize the sequence surrounding around this tyrosine and observed one preferred motif: AL**Y**DY/F. In 1992, Musacchio et al., aligned sequences of known SH3 domains in order to predict a possible tertiary structure of SH3 domain. Although the study badly proposed a presence of a long β-strand at the N-terminus of the SH3 domain, the ALYDY motif was described to be especially conserved (Musacchio et al., 1992). In combination with previous observation, the two tyrosines of ALYDY motif suggested phosphorylation of these sites with further regulatory role on a concrete protein function (Grandori, 1989; Kato et al., 1986; Potts et al., 1988; Yonemoto et al., 1985). On the other hand the two tyrosines were not strictly invariant in SH3 domain and were often substituted for phenylalanines (Musacchio et al., 1992) which actually resembles our results when considering position Y/F9. This raises a suggestion that the SH3 domains with ALYDY/F motif are divided into two groups: the ones that could be phosphorylated on position Y9 and the ones that have this tyrosine substituted for a non-phosphorylatable amino acid. This notion also highlights the significance of Y/F9 that is also one of the residues on the binding surface of the SH3 domain (Erpel et al., 1995). When looking at the set of all human SH3 domains we recorded around 12% of sequences containing ALYDY or ALYDF motif at the N-terminal part of the domain that could be possibly phosphorylated. Although we realize that not all of the motifs will be phosphorylated since they do not have to be available for kinases and not always

the phosphorylating kinase have to be present. However, 12 ALYDY/F motifs were found to be phosphorylated so far and we can therefore expect more experimental evidence on the importance of Y7 phosphoryaltion in near future.

During our bioinformatic analysis on SH3 domain tyrosine phosphorylation we observed that proportion of tyrosine phosphorylation in SH3 domain is overrepresented when compared to ratio of all tyrosine phopshorylations in the database we used. Around 20% of tyrosines were described to be phosphorylated in the Phophosite Plus database but almost 70% of them when considering only the SH3 domains. Thus we were wondering whether we could see the same trend in other adaptor domains. This notion was true for all but PTB domain where none of three phosphorylations was on tyrosine residue. This suggests that tyrosine phosphorylation could also be an important regulatory mechanism for other adaptor domains. One explanation of this phenomenon could be provided by work of Fabian et al., that described the effect of serine- versus tyrosine-phosphorylation on the conformation of a synthetic tau peptide and showed that while phosphorylation of serine residue had no impact on the structure of non-phosphorylated tau peptide, phosphorylation of tyrosine resulted in considerable conformation changes (Fabian et al., 1994).

7 Conclusions

- We have prepared GST- and GFP- fused CCH domain constructs that were further used to study the anchorage mechanism of CAS to cell membrane through this domain.
- We optimized the conditions of CAS CCH domain purification and suggested that the 46-aa region prior to the exact four-helix bundle is essential for proper function of this domain.
- We showed that the long variant of CAS CCH domain interacts with CAS, FAK and vinculin. FAK and vinculin are the FA targeting proteins of CAS CCH domain.
- CAS CCH domain localizes to podosomes suggesting a possible role of this domain in other than focal-type of adhesions.
- By mass spectrometry we have identified already known but also new interacting partners to be interacting with CAS SH3 domain. Vinculin, a protein which connects integrins to the cytoskeleton is a likely candidate in targeting CAS to focal complexes through the interaction with CAS SH3 domain.
- Vinculin “neck” motif is responsible for the interaction with CAS.
- Phosphomimiking Y12E mutation of CAS SH3 domain reduces its interaction with vinculin.
- We used publicly available resources to analyze the SH3 domain tyrosine phosphorylation. The most abundant phosphorylation site occured at position Y7 with preferred sequence motif ALYDY/F.
- Survey of tyrosine phosphorylation effects on SH3 domain binding poperties showed that it often blocks or prevents interactions.
- We showed that tyrosine phosphorylation is enriched in SH3 and other docking domains of signalling proteins. Tyrosine phosphorylation of the docking domains appears to be a general regulatory mechanism of their ligand binding.

8 References

Abassi, Y.A., Rehn, M., Ekman, N., Alitalo, K., and Vuori, K. (2003).p130Cas Couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. J Biol Chem *278*, 35636-35643.

Abercrombie, M., Heaysman, J.E., and Pegrum, S.M. (1971).The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Exp Cell Res *67*, 359-367.

Akakura, S., Kar, B., Singh, S., Cho, L., Tibrewal, N., Sanokawa-Akakura, R., Reichman, C., Ravichandran, K.S., and Birge, R.B. (2005). C-terminal SH3 domain of CrkII regulates the assembly and function of the DOCK180/ELMO Rac-GEF. J Cell Physiol *204*, 344-351.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008). *Molecular Biology of the Cell.* Garland Science, USA. 5th edition. ISBN 978-0-8153-4106-2: 175.

Ben-Ze'ev, A., Robinson, G.S., Bucher, N.L., and Farmer, S.R. (1988). Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. Proc Natl Acad Sci U S A *85*, 2161-2165.

Bertolucci, C.M., Guibao, C.D., and Zheng, J. (2005). Structural features of the focal adhesion kinase-paxillin complex give insight into the dynamics of focal adhesion assembly. Protein Sci *14*, 644-652.

Bhaskara, R.M., and Srinivasan, N. (2011).Stability of domain structures in multi-domain proteins.Sci Rep *1*, 40.

Bockholt, S.M., and Burridge, K. (1993). Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. J Biol Chem *268*, 14565-14567.

Bouton, A.H., Riggins, R.B., and Bruce-Staskal, P.J. (2001). Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. Oncogene *20*, 6448-6458.

Brábek, J., Constancio, S.S., Shin, N.Y., Pozzi, A., Weaver, A.M., and Hanks, S.K. (2004). CAS promotes invasiveness of Src-transformed cells. Oncogene *23*, 7406-7415.

Brábek, J., Constancio, S.S., Siesser, P.F., Shin, N.Y., Pozzi, A., and Hanks, S.K. (2005). Crk-associated substrate tyrosine phosphorylation sites are critical for invasion and metastasis of SRC-transformed cells. Mol Cancer Res *3*, 307-315.

Brábek, J., Mierke, C.T., Rösel, D., Veselý, P., and Fabry, B. (2010).The role of the tissue microenvironment in the regulation of cancer cell motility and invasion. Cell Commun Signal *8*, 22.

Brazin, K.N., Fulton, D.B., and Andreotti, A.H. (2000).A specific intermolecular association between the regulatory domains of a Tec family kinase. J Mol Biol *302*, 607-623.

Breheny, P.J., Laederach, A., Fulton, D.B., and Andreotti, A.H. (2003). Ligand specificity modulated by prolyl imide bond Cis/Trans isomerization in the Itk SH2 domain: a quantitative NMR study. J Am Chem Soc *125*, 15706-15707.

Brinkman, A., van der Flier, S., Kok, E.M., and Dorssers, L.C. (2000).BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. J Natl Cancer Inst *92*, 112-120.

Broome, M.A., and Hunter, T. (1996).Requirement for c-Src catalytic activity and the SH3 domain in platelet-derived growth factor BB and epidermal growth factor mitogenic signalling. J Biol Chem *271*, 16798-16806.

Buensuceso, C.S., and O'Toole, T.E. (2000). The association of CRKII with C3G can be regulated by integrins and defines a novel means to regulate the mitogen-activated protein kinases. J Biol Chem *275*, 13118-13125.

Burnham, M.R., Harte, M.T., Richardson, A., Parsons, J.T., and Bouton, A.H. (1996).The identification of p130cas-binding proteins and their role in cellular transformation.Oncogene *12*, 2467-2472.

Burridge, K., Turner, C.E., and Romer, L.H. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol *119*, 893-903.

Cabodi, S., del Pilar Camacho-Leal, M., Di Stefano, P., and Defilippi, P. (2010). Integrin signalling adaptors: not only figurants in the cancer story. Nat Rev Cancer *10*, 858-870.

Cabodi, S., Moro, L., Baj, G., Smeriglio, M., Di Stefano, P., Gippone, S., Surico, N., Silengo, L., Turco, E., Tarone, G., *et al.* (2004).p130Cas interacts with estrogen receptor alpha and modulates non-genomic estrogen signalling in breast cancer cells. J Cell Sci *117*, 1603-1611.

Cai, D., Clayton, L.K., Smolyar, A., and Lerner, A. (1999). AND-34, a novel p130Cas-binding thymic stromal cell protein regulated by adhesion and inflammatory cytokines. J Immunol *163*, 2104-2112.

Cai, D., Iyer, A., Felekkis, K.N., Near, R.I., Luo, Z., Chernoff, J., Albanese, C., Pestell, R.G., and Lerner, A. (2003). AND-34/BCAR3, a GDP exchange factor whose overexpression confers antiestrogen resistance, activates Rac, PAK1, and the cyclin D1 promoter. Cancer Res *63*, 6802-6808.

Calderwood, D.A. (2004) Integrin activation.,*J Cell Sci*, **117**, 657-666.

Cary, L.A., Han, D.C., Polte, T.R., Hanks, S.K., and Guan, J.L. (1998).Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J Cell Biol *140*, 211-221.

Casamassima, A., and Rozengurt, E. (1997). Tyrosine phosphorylation of p130(cas) by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. Signalling pathways and formation of a p130(cas)-Crk complex. J Biol Chem *272*, 9363-9370.

Castagnoli, L., Costantini, A., Dall'Armi, C., Gonfloni, S., Montecchi-Palazzi, L., Panni, S., Paoluzi, S., Santonico, E., and Cesareni, G. (2004). Selectivity and promiscuity in the interaction network mediated by protein recognition modules. FEBS Lett *567*, 74-79.

Chan, P.C., Lai, J.F., Cheng, C.H., Tang, M.J., Chiu, C.C., and Chen, H.C. (1999).Suppression of ultraviolet irradiation-induced apoptosis by overexpression of focal adhesion kinase in Madin-Darby canine kidney cells. J Biol Chem *274*, 26901-26906.

Chen, S., O'Reilly, L.P., Smithgall, T.E., and Engen, J.R. (2008). Tyrosine phosphorylation in the SH3 domain disrupts negative regulatory interactions within the c-Abl kinase core. J Mol Biol *383*, 414-423.

Cheng, C.H., Yu, K.C., Chen, H.L., Chen, S.Y., Huang, C.H., Chan, P.C., Wung, C.W., and Chen, H.C. (2004). Blockade of v-Src-stimulated tumor formation by the Src homology 3 domain of Crk-associated substrate (Cas). FEBS Lett *557*, 221-227.

Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol *133*, 1403-1415.

Cicchetti, P., Mayer, B.J., Thiel, G., and Baltimore, D. (1992).Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. Science *257*, 803-806.

Cohen, P. (2000). The regulation of protein function by multisite phosphorylation--a 25 year update. Trends Biochem Sci *25*, 596-601.

Consortium, U. (2012). Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res *40*, D71-75.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: a sequence logo generator. Genome Res *14*, 1188-1190.

Cunningham-Edmondson, A.C., and Hanks, S.K. (2009).p130Cas substrate domain signalling promotes migration, invasion, and survival of estrogen receptor-negative breast cancer cells. Breast Cancer (London) *2009*, 39-52.

Defilippi, P., Di Stefano, P., and Cabodi, S. (2006). p130Cas: a versatile scaffold in signalling networks. Trends Cell Biol *16*, 257-263.

del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M., and Sheetz, M.P. (2009). Stretching single talin rod molecules activates vinculin binding. Science *323*, 638-641.

Di Stefano, P., Cabodi, S., Boeri Erba, E., Margaria, V., Bergatto, E., Giuffrida, M.G., Silengo, L., Tarone, G., Turco, E., and Defilippi, P. (2004).P130Cas-associated protein (p140Cap) as a new tyrosine-phosphorylated protein involved in cell spreading. Mol Biol Cell *15*, 787-800.

Di Stefano, P., Damiano, L., Cabodi, S., Aramu, S., Tordella, L., Praduroux, A., Piva, R., Cavallo, F., Forni, G., Silengo, L., *et al.* (2007).p140Cap protein suppresses tumour cell properties, regulating Csk and Src kinase activity. EMBO J *26*, 2843-2855.

Donato, D.M., Ryzhova, L.M., Meenderink, L.M., Kaverina, I., and Hanks, S.K. (2010).Dynamics and mechanism of p130Cas localization to focal adhesions. J Biol Chem *285*, 20769-20779.

Dorssers, L.C., Grebenchtchikov, N., Brinkman, A., Look, M.P., van Broekhoven, S.P., de Jong, D., Peters, H.A., Portengen, H., Meijer-van Gelder, M.E., Klijn, J.G., *et al.* (2004). The prognostic value of BCAR1 in patients with primary breast cancer. Clin Cancer Res *10*, 6194-6202.

Eckhart, W., Hutchinson, M.A., and Hunter, T. (1979).An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell *18*, 925-933.

Erpel, T., Superti-Furga, G., and Courtneidge, S.A. (1995). Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J *14*, 963-975.

Evans, E.A., and Calderwood, D.A. (2007). Forces and bond dynamics in cell adhesion. Science *316*, 1148-1153.

Fabian, H., Otvos, L., Szendrei, G.I., Lang, E., and Mantsch, H.H. (1994). Tyrosine- versus serine-phosphorylation leads to conformational changes in a synthetic tau peptide. J Biomol Struct Dyn *12*, 573-579.

Fan, Z., Tardif, G., Hum, D., Duval, N., Pelletier, J.P., and Martel-Pelletier, J. (2009).Hsp90{beta} and p130(cas): novel regulatory factors of MMP-13 expression in human osteoarthritic chondrocytes. Ann Rheum Dis *68*, 976-982.

Feng, S., Kasahara, C., Rickles, R.J., and Schreiber, S.L. (1995). Specific interactions outside the proline-rich core of two classes of Src homology 3 ligands. Proc Natl Acad Sci U S A *92*, 12408-12415.

Fernow, I., Tomasovic, A., Siehoff-Icking, A., and Tikkanen, R. (2009). Cbl-associated protein is tyrosine phosphorylated by c-Abl and c-Src kinases. BMC Cell Biol *10*, 80.

Fielding, A.B., Dobreva, I., and Dedhar, S. (2008). Beyond focal adhesions: integrin-linked kinase associates with tubulin and regulates mitotic spindle organization. Cell Cycle *7*, 1899-1906.

Finn, R.D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J.E., Gavin, O.L., Gunasekaran, P., Ceric, G., Forslund, K., *et al.* (2010). The Pfam protein families database. Nucleic Acids Res *38*, D211-222.

Fischer, E.H., and Krebs, E.G. (1955). Conversion of phosphorylase b to phosphorylase a in muscle extracts. J Biol Chem *216*, 121-132.

Fonseca, P.M., Shin, N.Y., Brábek, J., Ryzhova, L., Wu, J., and Hanks, S.K. (2004).Regulation and localization of CAS substrate domain tyrosine phosphorylation. Cell Signal *16*, 621-629.

Friedland, J.C., Lee, M.H., and Boettiger, D. (2009). Mechanically activated integrin switch controls alpha5beta1 function. Science *323*, 642-644.

Garron, M.L., Arsenieva, D., Zhong, J., Bloom, A.B., Lerner, A., O'Neill, G.M., and Arold, S.T. (2009). Structural insights into the association between BCAR3 and Cas family members, an atypical complex implicated in anti-oestrogen resistance. J Mol Biol *386*, 190-203.

Garton, A.J., Flint, A.J., and Tonks, N.K. (1996). Identification of p130(cas) as a substrate for the cytosolic protein tyrosine phosphatase PTP-PEST. Mol Cell Biol *16*, 6408-6418.

Geiger, B., and Bershadsky, A. (2002).Exploring the neighborhood: adhesion-coupled cell mechanosensors. Cell *110*, 139-142.

Gilmore, T.D., Radke, K., and Martin, G.S. (1982). Tyrosine phosphorylation of a 50K cellular polypeptide associated with the Rous sarcoma virus transforming protein pp60src. Mol Cell Biol *2*, 199-206.

Gotoh, T., Cai, D., Tian, X., Feig, L.A., and Lerner, A. (2000). p130Cas regulates the activity of AND-34, a novel Ral, Rap1, and R-Ras guanine nucleotide exchange factor. J Biol Chem *275*, 30118-30123.

Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., and Takahashi, H. (1995).Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G.Mol Cell Biol *15*, 6746-6753.

Gough, J., Karplus, K., Hughey, R., and Chothia, C. (2001). Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol *313*, 903-919

Grandori, C. (1989). Regulation of kinase activity.Nature *338*, 467.

Grashoff, C., Hoffman, B.D., Brenner, M.D., Zhou, R., Parsons, M., Yang, M.T., McLean, M.A., Sligar, S.G., Chen, C.S., Ha, T., *et al.* (2010). Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. Nature *466*, 263-266.

Guan, J.L., and Shalloway, D. (1992).Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature *358*, 690-692.

Han, J.H., Batey, S., Nickson, A.A., Teichmann, S.A., and Clarke, J. (2007).The folding and evolution of multidomain proteins. Nat Rev Mol Cell Biol *8*, 319-330.

Hannigan, G.E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M.G., Radeva, G., Filmus, J., Bell, J.C., and Dedhar, S. (1996). Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature *379*, 91-96.

Hansson, H., Mattsson, P.T., Allard, P., Haapaniemi, P., Vihinen, M., Smith, C.I., and Hard, T. (1998).Solution structure of the SH3 domain from Bruton's tyrosine kinase.Biochemistry *37*, 2912-2924.

Harte, M.T., Hildebrand, J.D., Burnham, M.R., Bouton, A.H., and Parsons, J.T. (1996). p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J Biol Chem *271*, 13649-13655.

Harte, M.T., Macklem, M., Weidow, C.L., Parsons, J.T., and Bouton, A.H. (2000). Identification of two focal adhesion targeting sequences in the adapter molecule p130(Cas). Biochim Biophys Acta *1499*, 34-48.

Hayashi, I., Vuori, K., and Liddington, R.C. (2002). The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. Nat Struct Biol *9*, 101-106.

Hoellerer, M.K., Noble, M.E., Labesse, G., Campbell, I.D., Werner, J.M., and Arold, S.T. (2003). Molecular recognition of paxillin LD motifs by the focal adhesion targeting domain. Structure *11*, 1207-1217.

Hofmann, K. (1999). The modular nature of apoptotic signalling proteins. Cell Mol Life Sci *55*, 1113-1128.

Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., *et al.* (1998).Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat Genet *19*, 361-365.

Hornbeck, P.V., Chabra, I., Kornhauser, J.M., Skrzypek, E., and Zhang, B. (2004). PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation. Proteomics *4*, 1551-1561.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C., and Burridge, K. (1986).Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. Nature *320*, 531-533.

Humphries, J.D., Byron, A., and Humphries, M.J. (2006).Integrin ligands at a glance.J Cell Sci *119*, 3901-3903.

Hunter, T. (2009). Tyrosine phosphorylation: thirty years and counting. Curr Opin Cell Biol *21*, 140-146.

Hunter, T., and Sefton, B.M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci U S A *77*, 1311-1315.

Hynes, R.O. (2002) A reevaluation of integrins as regulators of angiogenesis.,*Nat Med*, **8**, 918-921.

Ichijo, H. (1999). From receptors to stress-activated MAP kinases.Oncogene *18*, 6087-6093.

Ingham, R.J., Krebs, D.L., Barbazuk, S.M., Turck, C.W., Hirai, H., Matsuda, M., and Gold, M.R. (1996). B cell antigen receptor signalling induces the formation of complexes containing the Crk adapter proteins. J Biol Chem *271*, 32306-32314.

Ishino, M., Ohba, T., Sasaki, H., and Sasaki, T. (1995).Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn.Oncogene *11*, 2331-2338.

Janostiak, R., Tolde, O., Bruhová, Z., Novotny, M., Hanks, S.K., Rösel, D., and Brábek, J. (2011). Tyrosine phosphorylation within the SH3 domain regulates CAS subcellular localization, cell migration, and invasiveness. Mol Biol Cell *22*, 4256-4267.

Joseph, R.E., Fulton, D.B., and Andreotti, A.H. (2007).Mechanism and functional significance of Itk autophosphorylation. J Mol Biol *373*, 1281-1292.

Kaneko, T., Li, L., and Li, S.S. (2008).The SH3 domain--a family of versatile peptide- and protein-recognition module. Front Biosci *13*, 4938-4952.

Kang, Y.S., Kim, W., Huh, Y.H., Bae, J., Kim, J.S., and Song, W.K. (2011). P130Cas attenuates epidermal growth factor (EGF) receptor internalization by modulating EGF-triggered dynamin phosphorylation. PLoS One *6*, e20125.

Kanner, S.B., Reynolds, A.B., Wang, H.C., Vines, R.R., and Parsons, J.T. (1991). The SH2 and SH3 domains of pp60src direct stable association with tyrosine phosphorylated proteins p130 and p110. EMBO J *10*, 1689-1698.

Kashiwakura, J., Suzuki, N., Takeno, M., Itoh, S., Oku, T., Sakane, T., Nakajin, S., and Toyoshima, S. (2002). Evidence of autophosphorylation in Txk: Y91 is an autophosphorylation site. Biol Pharm Bull *25*, 718-721.

Kato, J.Y., Takeya, T., Grandori, C., Iba, H., Levy, J.B., and Hanafusa, H. (1986).Amino acid substitutions sufficient to convert the nontransforming p60c-src protein to a transforming protein.Mol Cell Biol *6*, 4155-4160.

Katoh, H., and Negishi, M. (2003). RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature *424*, 461-464.

Kim, W., Kook, S., Kim, D.J., Teodorof, C., and Song, W.K. (2004). The 31-kDa caspase-generated cleavage product of p130cas functions as a transcriptional repressor of E2A in apoptotic cells. J Biol Chem *279*, 8333-8342.

Kirsch, K.H., Georgescu, M.M., and Hanafusa, H. (1998). Direct binding of p130(Cas) to the guanine nucleotide exchange factor C3G. J Biol Chem *273*, 25673-25679.

Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998). Activation of Rac1 by a Crk SH3-binding protein, DOCK180.Genes Dev *12*, 3331-3336.

Klemke, R.L., Leng, J., Molander, R., Brooks, P.C., Vuori, K., and Cheresh, D.A. (1998). CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol *140*, 961-972.

Klinghoffer, R.A., Sachsenmaier, C., Cooper, J.A., and Soriano, P. (1999). Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J *18*, 2459-2471.

Kook, S., Shim, S.R., Choi, S.J., Ahnn, J., Kim, J.I., Eom, S.H., Jung, Y.K., Paik, S.G., and Song, W.K. (2000).Caspase-mediated cleavage of p130cas in etoposide-induced apoptotic Rat-1 cells. Mol Biol Cell *11*, 929-939.

Kopfstein, L., and Christofori, G. (2006). Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment. Cell Mol Life Sci *63*, 449-468.

Kuriyan, J., and Cowburn, D. (1997).Modular peptide recognition domains in eukaryotic signalling. Annu Rev Biophys Biomol Struct *26*, 259-288.

Law, S.F., Estojak, J., Wang, B., Mysliwiec, T., Kruh, G., and Golemis, E.A. (1996). Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae. Mol Cell Biol *16*, 3327-3337.

Law, S.F., Zhang, Y.Z., Fashena, S.J., Toby, G., Estojak, J., and Golemis, E.A. (1999).Dimerization of the docking/adaptor protein HEF1 via a carboxy-terminal helix-loop-helix domain. Exp Cell Res *252*, 224-235.

Lazer, G., Pe'er, L., Farago, M., Machida, K., Mayer, B.J., and Katzav, S. (2010). Tyrosine residues at the carboxyl terminus of Vav1 play an important role in regulation of its biological activity. J Biol Chem *285*, 23075-23085.

Lee, C.H., Leung, B., Lemmon, M.A., Zheng, J., Cowburn, D., Kuriyan, J., and Saksela, K. (1995). A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. EMBO J *14*, 5006-5015.

Lenter, M., and Vestweber, D. (1994). The integrin chains beta 1 and alpha 6 associate with the chaperone calnexin prior to integrin assembly. J Biol Chem *269*, 12263-12268.

Letunic, I., Doerks, T., and Bork, P. (2009). SMART 6: recent updates and new developments. Nucleic Acids Res *37*, D229-232.

Li, L., Okura, M., and Imamoto, A. (2002). Focal adhesions require catalytic activity of Src family kinases to mediate integrin-matrix adhesion. Mol Cell Biol *22*, 1203-1217.

Li, S., Couvillon, A.D., Brasher, B.B., and Van Etten, R.A. (2001). Tyrosine phosphorylation of Grb2 by Bcr/Abl and epidermal growth factor receptor: a novel regulatory mechanism for tyrosine kinase signalling. EMBO J *20*, 6793-6804.

Lim, W.A., and Pawson, T. (2010). Phosphotyrosine signalling: evolving a new cellular communication system. Cell *142*, 661-667.

Liu, F., Sells, M.A., and Chernoff, J. (1998). Transformation suppression by protein tyrosine phosphatase 1B requires a functional SH3 ligand. Mol Cell Biol *18*, 250-259.

Lulo, J., Yuzawa, S., and Schlessinger, J. (2009). Crystal structures of free and ligand-bound focal adhesion targeting domain of Pyk2. Biochem Biophys Res Commun *383*, 347-352.

Luo, B.H., Carman, C.V., and Springer, T.A. (2007).Structural basis of integrin regulation and signalling. Annu Rev Immunol *25*, 619-647.

Luo, W., Slebos, R.J., Hill, S., Li, M., Brábek, J., Amanchy, R., Chaerkady, R., Pandey, A., Ham, A.J., and Hanks, S.K. (2008).Global impact of oncogenic Src on a phosphotyrosine proteome. J Proteome Res *7*, 3447-3460.

Mace, P.D., Wallez, Y., Dobaczewska, M.K., Lee, J.J., Robinson, H., Pasquale, E.B., and Riedl, S.J. (2011). NSP-Cas protein structures reveal a promiscuous interaction module in cell signalling. Nat Struct Mol Biol *18*, 1381-1387.

Makkinje, A., Near, R.I., Infusini, G., Vanden Borre, P., Bloom, A., Cai, D., Costello, C.E., and Lerner, A. (2009). AND-34/BCAR3 regulates adhesion-dependent p130Cas serine phosphorylation and breast cancer cell growth pattern. Cell Signal *21*, 1423-1435.

Mayer, B.J. (2001). SH3 domains: complexity in moderation. J Cell Sci *114*, 1253-1263.

Mayer, B.J., Hamaguchi, M., and Hanafusa, H. (1988).A novel viral oncogene with structural similarity to phospholipase C. Nature *332*, 272-275.

Mayer, B.J., Jackson, P.K., and Baltimore, D. (1991). The noncatalytic src homology region 2 segment of abl tyrosine kinase binds to tyrosine-phosphorylated cellular proteins with high affinity. Proc Natl Acad Sci U S A *88*, 627-631.

McClay, D.R., and Ettensohn, C.A. (1987).Cell adhesion in morphogenesis. Annu Rev Cell Biol *3*, 319-345.

Meyn, M.A., Wilson, M.B., Abdi, F.A., Fahey, N., Schiavone, A.P., Wu, J., Hochrein, J.M., Engen, J.R., and Smithgall, T.E. (2006). Src family kinases phosphorylate the Bcr-Abl SH3-SH2 region and modulate Bcr-Abl transforming activity. J Biol Chem *281*, 30907-30916.

Miranti, C.K. and Brugge, J.S. (2002) Sensing the environment: a historical perspective on integrin signal transduction.,*Nat Cell Biol*, **4**, E83-90.

Miyamoto, S., Teramoto, H., Coso, O.A., Gutkind, J.S., Burbelo, P.D., Akiyama, S.K., and Yamada, K.M. (1995). Integrin function: molecular hierarchies of cytoskeletal and signalling molecules. J Cell Biol *131*, 791-805.

Miyamoto, S., Teramoto, H., Gutkind, J.S., and Yamada, K.M. (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol *135*, 1633-1642.

Moro, L., Dolce, L., Cabodi, S., Bergatto, E., Boeri Erba, E., Smeriglio, M., Turco, E., Retta, S.F., Giuffrida, M.G., Venturino, M., *et al.* (2002). Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J Biol Chem *277*, 9405-9414.

Morrogh, L.M., Hinshelwood, S., Costello, P., Cory, G.O., and Kinnon, C. (1999). The SH3 domain of Bruton's tyrosine kinase displays altered ligand binding properties when auto-phosphorylated in vitro. Eur J Immunol *29*, 2269-2279.

Moschos, S.J., Drogowski, L.M., Reppert, S.L., and Kirkwood, J.M. (2007).Integrins and cancer. Oncology (Williston Park) *21*, 13-20.

Musacchio, A., Gibson, T., Lehto, V.P., and Saraste, M. (1992).SH3--an abundant protein domain in search of a function. FEBS Lett *307*, 55-61.

Nagar, B., Hantschel, O., Seeliger, M., Davies, J.M., Weis, W.I., Superti-Furga, G., and Kuriyan, J. (2006).Organization of the SH3-SH2 unit in active and inactive forms of the c-Abl tyrosine kinase. Mol Cell *21*, 787-798.

Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell *112*, 859-871.

Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., and Hirai, H. (1997).Requirements for localization of p130cas to focal adhesions.Mol Cell Biol *17*, 3884-3897.

Nakamoto, T., Yamagata, T., Sakai, R., Ogawa, S., Honda, H., Ueno, H., Hirano, N., Yazaki, Y., and Hirai, H. (2000). CIZ, a zinc finger protein that interacts with p130(cas) and activates the expression of matrix metalloproteinases. Mol Cell Biol *20*, 1649-1658.

Nojima, Y., Morino, N., Mimura, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., and Yazaki, Y. (1995). Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130Cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. J Biol Chem *270*, 15398-15402.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J., *et al.* (2001). Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis.EMBO J *20*, 3333-3341.

Oktay, M., Wary, K.K., Dans, M., Birge, R.B., and Giancotti, F.G. (1999). Integrin-mediated activation of focal adhesion kinase is required for signalling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. J Cell Biol *145*, 1461-1469.

O'Neill, G.M., Fashena, S.J., and Golemis, E.A. (2000). Integrin signalling: a new Cas(t) of characters enters the stage. Trends Cell Biol *10*, 111-119.

Otey, C.A., Pavalko, F.M., and Burridge, K. (1990).An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. J Cell Biol *111*, 721-729.

Park, H., Wahl, M.I., Afar, D.E., Turck, C.W., Rawlings, D.J., Tam, C., Scharenberg, A.M., Kinet, J.P., and Witte, O.N. (1996).Regulation of Btk function by a major autophosphorylation site within the SH3 domain. Immunity *4*, 515-525.

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., *et al.* (2005).Tensional homeostasis and the malignant phenotype. Cancer Cell *8*, 241-254.

Pawson, T. (1988). Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene *3*, 491-495.

Pawson, T. (2004). Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell *116*, 191-203.

Pawson, T., and Nash, P. (2003).Assembly of cell regulatory systems through protein interaction domains. Science *300*, 445-452.

Petit, V., and Thiery, J.P. (2000). Focal adhesions: structure and dynamics. Biol Cell *92*, 477-494.

Petruzzelli, L., Takami, M., and Herrera, R. (1996). Adhesion through the interaction of lymphocyte function-associated antigen-1 with intracellular adhesion molecule-1 induces tyrosine phosphorylation of p130cas and its association with c-CrkII. J Biol Chem *271*, 7796-7801.

Plopper, G.E., McNamee, H.P., Dike, L.E., Bojanowski, K., and Ingber, D.E. (1995).Convergence of integrin and growth factor receptor signalling pathways within the focal adhesion complex. Mol Biol Cell *6*, 1349-1365.

Podar, K., Tai, Y.T., Lin, B.K., Narsimhan, R.P., Sattler, M., Kijima, T., Salgia, R., Gupta, D., Chauhan, D., and Anderson, K.C. (2002). Vascular endothelial growth factor-induced migration of multiple myeloma cells is associated with beta 1 integrin- and phosphatidylinositol 3-kinase-dependent PKC alpha activation. J Biol Chem *277*, 7875-7881.

Polte, T.R., and Hanks, S.K. (1995).Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc Natl Acad Sci U S A *92*, 10678-10682.

Potts, W.M., Reynolds, A.B., Lansing, T.J., and Parsons, J.T. (1988).Activation of pp60c-src transforming potential by mutations altering the structure of an amino terminal domain containing residues 90-95. Oncogene Res *3*, 343-355.

Prasad, N., Topping, R.S., and Decker, S.J. (2001). SH2-containing inositol 5'-phosphatase SHIP2 associates with the p130(Cas) adapter protein and regulates cellular adhesion and spreading. Mol Cell Biol *21*, 1416-1428.

Pratt, S.J., Epple, H., Ward, M., Feng, Y., Braga, V.M., and Longmore, G.D. (2005). The LIM protein Ajuba influences p130Cas localization and Rac1 activity during cell migration. J Cell Biol *168*, 813-824.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., *et al.* (2005).Global analysis of protein phosphorylation in yeast. Nature *438*, 679-684.

Qian, X., Li, G., Vass, W.C., Papageorge, A., Walker, R.C., Asnaghi, L., Steinbach, P.J., Tosato, G., Hunter, K., and Lowy, D.R. (2009). The Tensin-3 protein, including its SH2 domain, is phosphorylated by Src and contributes to tumorigenesis and metastasis. Cancer Cell *16*, 246-258.

Raftopoulou, M., and Hall, A. (2004). Cell migration: Rho GTPases lead the way. Dev Biol *265*, 23-32.

Ren, R., Mayer, B.J., Cicchetti, P., and Baltimore, D. (1993).Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157-1161.

Reynolds, A.B., Kanner, S.B., Wang, H.C., and Parsons, J.T. (1989).Stable association of activated pp60src with two tyrosine-phosphorylated cellular proteins.Mol Cell Biol *9*, 3951-3958.

Ribon, V., and Saltiel, A.R. (1996). Nerve growth factor stimulates the tyrosine phosphorylation of endogenous Crk-II and augments its association with p130Cas in PC-12 cells. J Biol Chem *271*, 7375-7380.

Riggins, R.B., Quilliam, L.A., and Bouton, A.H. (2003). Synergistic promotion of c-Src activation and cell migration by Cas and AND-34/BCAR3. J Biol Chem *278*, 28264-28273.

Riveline, D., Zamir, E., Balaban, N.Q., Schwarz, U.S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B., and Bershadsky, A.D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol *153*, 1175-1186.

Rojiani, M.V., Finlay, B.B., Gray, V., and Dedhar, S. (1991). In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits.Biochemistry *30*, 9859-9866.

Rolli, M., Fransvea, E., Pilch, J., Saven, A., and Felding-Habermann, B. (2003). Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. Proc Natl Acad Sci U S A *100*, 9482-9487.

Roselli, S., Wallez, Y., Wang, L., Vervoort, V., and Pasquale, E.B. (2010). The SH2 domain protein Shep1 regulates the in vivo signalling function of the scaffolding protein Cas. Cell Signal *22*, 1745-1752.

Ruest, P.J., Shin, N.Y., Polte, T.R., Zhang, X., and Hanks, S.K. (2001). Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. Mol Cell Biol *21*, 7641-7652.

Sadowski, I., Stone, J.C., and Pawson, T. (1986). A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. Mol Cell Biol *6*, 4396-4408.

Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994). A novel signalling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J *13*, 3748-3756.

Sakakibara, A., and Hattori, S. (2000). Chat, a Cas/HEF1-associated adaptor protein that integrates multiple signalling pathways.J Biol Chem *275*, 6404-6410.

Sakakibara, A., Ohba, Y., Kurokawa, K., Matsuda, M., and Hattori, S. (2002).Novel function of Chat in controlling cell adhesion via Cas-Crk-C3G-pathway-mediated Rap1 activation.J Cell Sci *115*, 4915-4924.

Sander, E.E., and Collard, J.G. (1999). Rho-like GTPases: their role in epithelial cell-cell adhesion and invasion. Eur J Cancer *35*, 1905-1911.

Sanes, J.R. (1989). Extracellular matrix molecules that influence neural development. Annu Rev Neurosci *12*, 491-516.

Sastry, S.K., Lyons, P.D., Schaller, M.D., and Burridge, K. (2002). PTP-PEST controls motility through regulation of Rac1. J Cell Sci *115*, 4305-4316.

Sato, M., Maruoka, M., Yokota, N., Kuwano, M., Matsui, A., Inada, M., Ogawa, T., Ishida-Kitagawa, N., and Takeya, T. (2011).Identification and functional analysis of a new phosphorylation site (Y398) in the SH3 domain of Abi-1. FEBS Lett *585*, 834-840.

Sawada, Y., Nakamura, K., Doi, K., Takeda, K., Tobiume, K., Saitoh, M., Morita, K., Komuro, I., De Vos, K., Sheetz, M., *et al.* (2001). Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase. J Cell Sci *114*, 1221-1227.

Sawada, Y., Tamada, M., Dubin-Thaler, B.J., Cherniavskaya, O., Sakai, R., Tanaka, S., and Sheetz, M.P. (2006). Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell *127*, 1015-1026.

Schaller, M.D., Hildebrand, J.D., and Parsons, J.T. (1999). Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. Mol Biol Cell *10*, 3489-3505.

Schaller, M.D., Otey, C.A., Hildebrand, J.D., and Parsons, J.T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J Cell Biol *130*, 1181-1187.

Schlaepfer, D.D., Broome, M.A., and Hunter, T. (1997). Fibronectin-stimulated signalling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol Cell Biol *17*, 1702-1713.

Schlaepfer, D.D., Jones, K.C., and Hunter, T. (1998). Multiple Grb2-mediated integrin-stimulated signalling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol Cell Biol *18*, 2571-2585.

Schuh, N.R., Guerrero, M.S., Schrecengost, R.S., and Bouton, A.H. (2010). BCAR3 regulates Src/p130 Cas association, Src kinase activity, and breast cancer adhesion signalling. J Biol Chem *285*, 2309-2317.

Schultz, J., Milpetz, F., Bork, P., and Ponting, C.P. (1998). SMART, a simple modular architecture research tool: identification of signalling domains. Proc Natl Acad Sci U S A *95*, 5857-5864.

Severin, A., Joseph, R.E., Boyken, S., Fulton, D.B., and Andreotti, A.H. (2009). Proline isomerization preorganizes the Itk SH2 domain for binding to the Itk SH3 domain. J Mol Biol *387*, 726-743.

Shah, R., Alvarez, M., Jones, D.R., Torrungruang, K., Watt, A.J., Selvamurugan, N., Partridge, N.C., Quinn, C.O., Pavalko, F.M., Rhodes, S.J., *et al.* (2004). Nmp4/CIZ regulation of matrix metalloproteinase 13 (MMP-13) response to parathyroid hormone in osteoblasts. Am J Physiol Endocrinol Metab *287*, E289-296.

Sharma, C.P., Ezzell, R.M., and Arnaout, M.A. (1995).Direct interaction of filamin (ABP-280) with the beta 2-integrin subunit CD18.J Immunol *154*, 3461-3470.

Singh, M.K., Dadke, D., Nicolas, E., Serebriiskii, I.G., Apostolou, S., Canutescu, A., Egleston, B.L., and Golemis, E.A. (2008). A novel Cas family member, HEPL, regulates FAK and cell spreading. Mol Biol Cell *19*, 1627-1636.

Soni, S., Lin, B.T., August, A., Nicholson, R.I., and Kirsch, K.H. (2009). Expression of a phosphorylated p130(Cas) substrate domain attenuates the phosphatidylinositol 3-kinase/Akt survival pathway in tamoxifen resistant breast cancer cells. J Cell Biochem *107*, 364-375.

Sparks, A.B., Rider, J.E., Hoffman, N.G., Fowlkes, D.M., Quillam, L.A., and Kay, B.K. (1996).Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCgamma, Crk, and Grb2. Proc Natl Acad Sci U S A *93*, 1540-1544.

Sriram, G., Reichman, C., Tunceroglu, A., Kaushal, N., Saleh, T., Machida, K., Mayer, B., Ge, Q., Li, J., Hornbeck, P., *et al.* (2011). Phosphorylation of Crk on tyrosine 251 in the RT loop of the SH3C domain promotes Abl kinase transactivation. Oncogene *30*, 4645-4655.

Stahl, M.L., Ferenz, C.R., Kelleher, K.L., Kriz, R.W., and Knopf, J.L. (1988).Sequence similarity of phospholipase C with the non-catalytic region of src. Nature *332*, 269-272.

Sturge, J., Hamelin, J., and Jones, G.E. (2002). N-WASP activation by a beta1-integrin-dependent mechanism supports PI3K-independent chemotaxis stimulated by urokinase-type plasminogen activator. J Cell Sci *115*, 699-711.

Sylvester, M., Kliche, S., Lange, S., Geithner, S., Klemm, C., Schlosser, A., Grossmann, A., Stelzl, U., Schraven, B., Krause, E., *et al.* (2010). Adhesion and degranulation promoting adapter protein (ADAP) is a central hub for phosphotyrosine-mediated interactions in T cells. PLoS One *5*, e11708.

Tachibana, K., Urano, T., Fujita, H., Ohashi, Y., Kamiguchi, K., Iwata, S., Hirai, H., and Morimoto, C. (1997). Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase.A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates. J Biol Chem *272*, 29083-29090.

Takino, T., Tamura, M., Miyamori, H., Araki, M., Matsumoto, K., Sato, H., and Yamada, K.M. (2003). Tyrosine phosphorylation of the CrkII adaptor protein modulates cell migration. J Cell Sci *116*, 3145-3155.

Tamada, M., Sheetz, M.P., and Sawada, Y. (2004).Activation of a signalling cascade by cytoskeleton stretch. Dev Cell *7*, 709-718.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res *22*, 4673-4680.

Tikhmyanova, N., Little, J.L., and Golemis, E.A. (2010). CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci *67*, 1025-1048.

Tolde, O., Rösel, D., Veselý, P., Folk, P., and Brábek, J. (2010).The structure of invadopodia in a complex 3D environment. Eur J Cell Biol *89*, 674-680.

Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G.A., Ladner, M., Long, C.M., Crosier, W.J., Watt, K., and Koths, K. (1988).Molecular cloning of two types of GAP complementary DNA from human placenta. Science *242*, 1697-1700.

Tsukamoto, N., Hattori, M., Yang, H., Bos, J.L., and Minato, N. (1999). Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion. J Biol Chem *274*, 18463-18469.

van Agthoven, T., van Agthoven, T.L., Dekker, A., van der Spek, P.J., Vreede, L., and Dorssers, L.C. (1998). Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells.EMBO J *17*, 2799-2808.

van Duijn, T.J., Anthony, E.C., Hensbergen, P.J., Deelder, A.M., and Hordijk, P.L. (2010). Rac1 recruits the adapter protein CMS/CD2AP to cell-cell contacts. J Biol Chem *285*, 20137-20146.

von Wichert, G., Jiang, G., Kostic, A., De Vos, K., Sap, J., and Sheetz, M.P. (2003). RPTP-alpha acts as a transducer of mechanical force on alphav/beta3-integrin-cytoskeleton linkages. J Cell Biol *161*, 143-153.

Vuori, K., and Ruoslahti, E. (1995). Tyrosine phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. J Biol Chem *270*, 22259-22262.

Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996). Introduction of p130cas signalling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Mol Cell Biol *16*, 2606-2613.

Weng, Z., Rickles, R.J., Feng, S., Richard, S., Shaw, A.S., Schreiber, S.L., and Brugge, J.S. (1995). Structure-function analysis of SH3 domains: SH3 binding specificity altered by single amino acid substitutions. Mol Cell Biol *15*, 5627-5634.

Wilcox, H.M., and Berg, L.J. (2003). Itk phosphorylation sites are required for functional activity in primary T cells. J Biol Chem *278*, 37112-37121.

Wisniewska, M., Bossenmaier, B., Georges, G., Hesse, F., Dangl, M., Künkele, K.P., Ioannidis, I., Huber, R., and Engh, R.A. (2005). The 1.1 A resolution crystal structure of the p130cas SH3 domain and ramifications for ligand selectivity. J Mol Biol *347*, 1005-1014.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U.H., Deryugina, E.I., Strongin, A.Y., Bröcker, E.B., and Friedl, P. (2003). Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol *160*, 267-277.

Wu, X., Gan, B., Yoo, Y., and Guan, J.L. (2005). FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. Dev Cell *9*, 185-196.

Wu, X., Knudsen, B., Feller, S.M., Zheng, J., Sali, A., Cowburn, D., Hanafusa, H., and Kuriyan, J. (1995).Structural basis for the specific interaction of lysine-containing proline-rich peptides with the N-terminal SH3 domain of c-Crk. Structure *3*, 215-226.

Wu, Y., Spencer, S.D., and Lasky, L.A. (1998). Tyrosine phosphorylation regulates the SH3-mediated binding of the Wiskott-Aldrich syndrome protein to PSTPIP, a cytoskeletal-associated protein. J Biol Chem *273*, 5765-5770.

Xu, W., Harrison, S.C., and Eck, M.J. (1997).Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

Yamakita, Y., Totsukawa, G., Yamashiro, S., Fry, D., Zhang, X., Hanks, S.K., and Matsumura, F. (1999). Dissociation of FAK/p130(CAS)/c-Src complex during mitosis: role of mitosis-specific serine phosphorylation of FAK. J Cell Biol *144*, 315-324.

Yonemoto, W., Jarvis-Morar, M., Brugge, J.S., Bolen, J.B., and Israel, M.A. (1985). Tyrosine phosphorylation within the amino-terminal domain of pp60c-src molecules associated with polyoma virus middle-sized tumor antigen. Proc Natl Acad Sci U S A *82*, 4568-4572.

Yoshigi, M., Hoffman, L.M., Jensen, C.C., Yost, H.J., and Beckerle, M.C. (2005). Mechanical force mobilizes zyxin from focal adhesions to actin filaments and regulates cytoskeletal reinforcement. J Cell Biol *171*, 209-215.

Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W., and Schreiber, S.L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell *76*, 933-945.

Yu, H., Rosen, M.K., Shin, T.B., Seidel-Dugan, C., Brugge, J.S., and Schreiber, S.L. (1992). Solution structure of the SH3 domain of Src and identification of its ligand-binding site. Science *258*, 1665-1668.

Zamir, E., and Geiger, B. (2001). Components of cell-matrix adhesions. J Cell Sci *114*, 3577-3579.

Zarrinpar, A., Bhattacharyya, R.P., and Lim, W.A. (2003).The structure and function of proline recognition domains.Sci STKE *2003*, RE8.

Zhang, Z., Vuori, K., Wang, H., Reed, J.C., and Ruoslahti, E. (1996).Integrin activation by R-ras. Cell *85*, 61-69.

Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A.M., and Burridge, K. (1998). Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. J Cell Biol *141*, 539-551.

Zhou, R., Huang, X., Margulis, C.J., and Berne, B.J. (2004).Hydrophobic collapse in multidomain protein folding. Science *305*, 1605-1609.

9 List of Supplements

1) Supporting data S5.1 – S5.12 and S6.1

2) Paper SH3 domain tyrosine phosphorylation – sites, role and evolution accepted to PLoS ONE Journal